UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

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MEMORANDUM

SUBJECT: Environmental Risk Assessment for a FIFRA Section 3 Registration of MON 89034 x TC1507 x MON 87411 x DAS-59122-7 Combined Trait Maize Expressing Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, Cry34/35Ab1 *Bacillus thuringiensis* Derived Insecticidal Protein, and DvSnf7 Double Stranded RNA (dsRNA); Submitted by Monsanto Company; EPA File Symbols 524-AGE, 524-AGR; PC Codes 006514, 006515, 006481, 006490, 006580, 006566; Decision Nos. 514588, 514589; Submission Nos. 982159, 983961, 982149, 985448; DP Barcodes: 432075, 433101, 432074, 433105; MRIDs 49748501, 49781805, 49781806, 49886501, 49886502, 49886503

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I. Introduction

Monsanto Company has submitted an application for FIFRA Section 3 registrations for MON 89034 x TC1507 x MON 87411 x DAS-59122-7 combined trait corn (EPA File Symbols 524-AGE and 524-AGR). MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn was developed through conventional breeding techniques, and contains multiple plant incorporated protectants (PIPs). PIPs are pesticidal substances produced by plants and the genetic material necessary for the plant to produce the substance. The PIPs in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn include several *Bacillus thuringiensis* (*Bt*) derived proteins and a double-stranded RNA (dsRNA) transcript, all of which confer resistance to certain insect pests. Specifically, MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn expresses Cry1A.105, Cry2Ab2, and Cry1F proteins for control of lepidopteran pests and Cry3Bb1, Cry34Ab1, Cry35Ab1 proteins for control of coleopteran pests. The double stranded RNA (dsRNA)

transcript, DvSnf7 dsRNA, is expressed by MON 87411 within this product. DvSnf7 dsRNA is derived from western corn rootworm (*Diabrotica virgifera virgifera*, WCR), and is included to target this pest. Additionally, MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn expresses the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) and the phosphinothricin acetyl transferase protein (PAT) from *Streptomyces viridochromogenes*, which confer tolerance to the herbicides, glyphosate and glufosinate-ammonium herbicides, respectively.

All of the PIPs included in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn have been registered in other single and/or combined trait products, and ecological risk assessments have been completed for each of these events. However, MON 87411 and the DvSnf7 dsRNA that it expresses were previously only registered for seed increase, so this proposed registration is for the first full commercial registration for this PIP. DvSnf7 provides a new and unique mode of action by utilizing RNA interference to downregulate an essential protein in the target pest, leading to mortality. Because of uncertainties related to dsRNA based pesticides, EPA limited its previous ecological risk assessment conclusions for DvSnf7 to the conditions of the seed increase registration, and required additional data to support a full commercial registration.

This risk assessment includes a review of these new data for MON 87411 and an update to the risk assessment conclusions for the full commercial registration of the DvSnf7 dsRNA expressed by this event. Therefore, this risk assessment is for the combined trait product, but focuses more specifically on the DvSnf7 dsRNA expressed in MON 87411. An assessment specific to DvSnf7 dsRNA is presented first below, followed by an assessment of the MON 89034 x TC1507 x MON 87411 x DAS-59122-7 combined trait product.

II. Ecological Risk Assessment for DvSnf7 dsRNA

A. General Approach to Ecological Risk Assessment for PIPs

EPA's current ecological risk assessment approach for PIPs was developed primarily from experience with *Bt*-derived Cry and Vip proteins. These proteins are generally understood to be specific to their target pests and related insects within the same taxonomic order, and with nearly two decades of history indicating safe use, EPA considers the current approach sufficient for determining ecological risks of *Bt*-derived protein PIPs.

This approach is described in several Biopesticide Registration Action Documents (BRADs) for Cry or Vip proteins (e.g., see U.S. EPA 2010a, pages 65-69). To summarize, the approach consists of a tiered testing scheme (Tiers I - IV) that is focused on hazard determination, and testing is based on the microbial pesticide data requirements published under 40 CFR 158.2150 and their associated 885 and 850 series OCSPP Harmonized Guidelines. At Tier I, studies are designed to be simplified and to estimate of hazard to several nontarget taxa under "worst-case" exposure conditions. A lack of adverse effects under these conditions would provide enough confidence that there is no risk and no further data would be needed. Screening (Tier I) maximum hazard dose tests are conducted at exposure concentrations several times higher (e.g., >10X when possible) than the highest concentrations expected to occur under realistic field exposure scenarios, with mortality as the toxicological endpoint. A threshold of 50% mortality is

used as a trigger for additional higher-tier testing. When screening studies suggest potentially unacceptable risk, additional studies are designed to assess hazard under more realistic field exposure conditions. Tiers II – IV generally encompass definitive hazard level determinations, chronic studies, and longer term greenhouse or field testing, and are implemented when unacceptable effects are seen at the Tier I screening level.

When screening tests indicate a need for additional data, the OCSPP Harmonized Guidelines call for testing at incrementally lower doses in order to establish a definitive LD_{50} and to quantify the hazard. A risk determination is made by comparing the toxicological endpoint to the estimated environmental concentration (EEC).

In addition to the toxicity data, additional data are also considered regarding the environmental persistence of PIP pesticidal substances, as well as the potential for gene flow and development of invasiveness. EPA requires laboratory data demonstrating the degradation of the PIP pesticidal substance in soils typical of agronomic areas where the PIP crop is grown. To assess gene flow and potential for development of invasiveness, EPA considers several lines of evidence related to characteristics of the crop plant, including reproduction, presence of wild relatives, and containment or other mitigating measures.

Because of uncertainties associated with dsRNA, EPA raised questions to the FIFRA Scientific Advisory Panel (SAP) regarding the applicability of the above approach to dsRNA based pesticides, including PIPs. The SAP's recommendations on alternatives to this framework are given in the minutes of the meeting (FIFRA SAP 2014). Those recommendations are discussed where applicable throughout this risk assessment, and also in Section I below.

B. DvSnf7 General Description and MON 87411 Regulatory Background

The DvSnf7 dsRNA expressed by event MON 87411 results from expression of an inverted repeat sequence designed to match the sequence of WCR *Snf7* gene. Expression of the sequence results in the formation of the dsRNA transcript containing a 240 bp fragment of the WCR *Snf7* (DvSnf7). Snf7 is a vacuolar sorting protein belonging to the Endosomal Sorting Complex Required for Transport (ESCRT)-III complex, which is involved in sorting of transmembrane proteins *en route* to lysosomal degradation through the endosomal-autophagic pathway. Once consumed, the DvSnf7 dsRNA is recognized by the WCR's RNA interference (RNAi) machinery wherein it is cleaved into 21-24 mer small interfering RNAs (siRNAs). These siRNAs bind to a RNA induced silencing complex (RISC), which ultimately leads to down-regulation of the targeted *DvSnf7* gene and mortality (MRID 48919004).

MON 87411 also expresses Cry3Bb1, which is a delta-endotoxin from *Bacillus thuringiensis* subsp. *kumamotoensis* that has specific activity against insects within the Order Coleoptera. Ecological risk associated with this protein is discussed below in Section III.

EPA issued an Experimental Use Permit (EUP) to Monsanto Company for MON 87411 and a similar event also expressing DvSnf7 (MON 87410) in 2013, and this EUP was to end in 2015. The EUP was extended in 2014 until February 2016, and was extended again in February 2015 for two additional years. A similar EUP was also issued to Dow AgroSciences, LLC in 2014,

which was extended in 2016 to March 2017. The ecological risk assessments for these EUPs relied primarily on data demonstrating the specificity of the DvSnf7 dsRNA for the WCR target pest as well as rationale describing barriers to uptake of the DvSnf7 dsRNA in nontarget organisms. In light of the limited acreage involving MON 87411 and MON 87410 and the limited duration, EPA determined that the risk of adverse effects to nontarget organisms was minimal.

Due to anticipated increased interest in registering dsRNA-based pesticide active ingredients and uncertainties related to this new technology, EPA consulted with the SAP for guidance on assessing risks from these pesticides. This guidance was sought with consideration of EPA's current risk assessment framework and the framework's ability to address uncertainties that were identified for dsRNA-based pesticides. The meeting of the SAP was held January 28, 2014, with minutes published in May of that year (FIFRA SAP 2014). The SAP consultation was held independently of any registration involving a dsRNA based pesticide, including MON 87411. However, advice provided to EPA by the SAP was expected to affect the risk assessment of MON 87411.

In October 2015, EPA granted a FIFRA Section 3 limited seed increase registration for MON 87411, which was limited to two years and restricted to 15,000 acres per year. Initially, Monsanto Company proposed this registration with the standard limits for a seed increase registration of 20,000 acres per county and up to a combined U.S. total of 250,000 acres per PIP active ingredient per year. In light of uncertainties discussed at the SAP meeting and recommendations of the SAP, EPA initially found the data supporting the original registration proposal to be deficient, and issued a deficiency letter during the course of review, which requested additional data. Monsanto Company submitted a response to the deficiency letter (MRID 49553302), which consisted of rationale that provided additional information from public literature to support a justification for no additional testing. Monsanto Company also requested the limitations to the proposed registration that are described above. EPA determined that the data submitted to support the ecological risk assessment for DvSnf7 in MON 87411 was sufficient for the limited registration, but that in light of uncertainties discussed at the SAP meeting additional data would be required to support the full commercial registration. These data are reviewed herein and are considered in the context of environmental exposure relevant to expression and environmental fate of DvSnf7 in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn.

C. Environmental Fate of DvSnf7 Expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn

Environmental fate for PIPs is largely influenced by expression levels of the PIP pesticidal substance within plant tissues and degradation of the PIP pesticidal substance within the environment. However, deposition and movement of the plant tissues in the environment; degradation of the PIP pesticidal substance within plant tissues; and deposition, movement, and degradation of the PIP pesticidal substance in the environment also affect environmental fate and exposure to nontarget organisms, and are considered in PIP risk assessments. The expression of DvSnf7 in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 is discussed below, along with its fate and resulting exposure estimates in terrestrial and aquatic environments.

1. Expression of DvSnf7 in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn

Field exposure rates are typically based on expression levels within the plant for PIP pesticidal substances. For the purposes of the non-target organism risk assessment of DvSnf7 in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn, estimated exposures are based on the highest concentration of these active ingredients expressed in corn tissue(s)/organ(s) based on expression studies. For Cry and Vip proteins, expression studies measure protein expression; for DvSnf7, the expression of the DvSnf7 dsRNA is measured. Fresh weight and dry weight measurements of the expressed product per gram of corn plant material are typically determined for PIP pesticidal substances; however, worst-case estimates of exposure are based on dry weight measurements, since fresh weight measurements can be highly variable (FIFRA SAP 2001).

For MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn, dry weight (dw) expression levels of DvSnf7 dsRNA in various plant organs and tissues collected from trials within the U.S. in 2013 are shown in Table 1. Expression in the single trait corn (MON 87411) from organs and tissues collected in the same trials is also shown for comparison. Expression levels were determined using the QuantiGene® Plex 2.0 Assay (Affymetrix, Inc.; Santa Clara, California), and the full analysis is described in MRID 49781804. Review of these data is given in USEPA (2016). Expression of DvSnf7 in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn is expected to be comparable to its expression in the MON 87411 single trait corn, and this expectation is supported by the data shown for each type of corn tissue/organs in Table 1.

The data from the U.S. trials do not show the trend in exposure over the season, which is important to the ecological risk assessment. A lack of trend data limits EPA's understanding of the fate of DvSnf7 over the growing season and beyond. However, such trend data were collected for DvSnf7 expression in MON 87411 in trials conducted in Argentina in 2011-2012. These data are shown in Table 2. Results of expression measured at similar growth stages as given in Table 1 are shown in boldface type, and show general agreement in expression levels with minor variation. In the Argentina trials, the highest expression was observed early in the season with measurements on the whole plant. Similar data were not provided for either the single- or combined-trait corn for the U.S. trials, but trends in expression over time are expected to be similar. The highest expression for MON 89034 x TC1507 x MON 87411 x DAS-15822-7 and MON 87411 corn in U.S. trials was seen in leaves early in the season, based on the data provided. These values may be used as an estimate of highest expression in the combined-trait corn; however, without a full set of data, there is some uncertainty that early season measurements on whole plants may be higher. Similarly, while the U.S. trials showed very little expression in pollen, measurable expression was observed in pollen in 25% (5 out of 20) of the samples taken in the Argentina trials. Therefore, an assumption that DvSnf7 is not expressed at all in MON 89034 x TC1507 x MON 87411 x DAS-15822-7 pollen is premature, although, if expressed, it is expected at very low levels relative to other plant tissues.

The data in Table 2 also show a general trend of decreasing expression over the growing season. After senescence, expression is approximately 17 times less than the highest expression during the growing season when comparing mean values for Over Season Root (1) to Senescent Root,

and approximately 130 times less when comparing mean values for Over Season Whole Plant (1) to Stover.

Table 1. DvSnf7 dsRNA expression levels in organs and tissues collected from MON 89034 x TC1507 x MON 87411 x DAS-59122-7 and MON 87411 corn plants in U.S. trials in 2013.

		Mean µg DvSnf7 dsRNA/g dw tissue ± SD (Range)	
Tissue Type	**Crop Development Stage[a]**	**MON 89034 x TC1507 x MON 87411 x DAS-59122-7[b]**	**MON 87411[c]**
Over Season Leaf	V2 - V4	$89 \times 10^{-3} \pm 25 \times 10^{-3}$ (41×10^{-3} - 132×10^{-3})	$97 \times 10^{-3} \pm 29 \times 10^{-3}$ (53×10^{-3} - 151×10^{-3})
Over Season Root	V2 - V4	$28 \times 10^{-3} \pm 15 \times 10^{-3}$ (10×10^{-3} - 58×10^{-3})	$32 \times 10^{-3} \pm 14 \times 10^{-3}$ (11×10^{-3} - 59×10^{-3})
Over Season Whole Plant	V10 - V11	$25 \times 10^{-3} \pm 5.5 \times 10^{-3}$ (16×10^{-3} - 37×10^{-3})	$26 \times 10^{-3} \pm 7.7 \times 10^{-3}$ (17×10^{-3} - 50×10^{-3})
Pollen	R1	N/A[d]	0.52×10^{-3} [e] (N/A)[e]
Forage	R5	$3.2 \times 10^{-3} \pm 0.82 \times 10^{-3}$ (1.7×10^{-3} - 4.4×10^{-3})	$4.0 \times 10^{-3} \pm 1.4 \times 10^{-3}$ (0.90×10^{-3} - 6.2×10^{-3})
Forage Root	R5	$1.9 \times 10^{-3} \pm 1.2 \times 10^{-3}$ (0.65×10^{-3} - 5.8×10^{-3})	$2.3 \times 10^{-3} \pm 1.7 \times 10^{-3}$ (0.57×10^{-3} - 6.1×10^{-3})
Grain	R6	$0.089 \times 10^{-3} \pm 0.032 \times 10^{-3}$ (0.045×10^{-3} - 0.15×10^{-3})	$0.084 \times 10^{-3} \pm 0.039 \times 10^{-3}$ (0.033×10^{-3} - 0.18×10^{-3})

[a] Crop development stages at which each tissue was collected.

[b] Means, SDs, and ranges calculated from data collected at 5 sites (n=20, except Over Season Root where n=16 due to unexpected result in four samples, and pollen where n=0)

[c] Means, SDs, and ranges calculated from data collected at 5 sites (n=20, except Over Season Whole Plant where n=19 due to unexpected result, and pollen where n = 1)

[d] N/A = not applicable; expression in all samples was below Limit of Quantitation (0.29×10^{-4} µg/g fresh weight) or Limit of Detection (0.065×10^{-4} µg/g fresh weight)

[e] n=19; expression in 19 of 20 samples was below Limit of Quantitation (0.24×10^{-4} µg/g fresh weight) or Limit of Detection (0.056×10^{-4} µg/g fresh weight)

Table 2. DvSnf7 dsRNA expression levels in tissues/organs collected from MON 87411 corn plants in trials Argentina, 2011-2012.

Tissue Type	Crop Development Stage	Mean µg DvSnf7 dsRNA/g dw tissue ± SD (Range)
Over Season Leaf (1)	**V3 - V4**	**$73.9 \times 10^{-3} \pm 14.5 \times 10^{-3}$ (43.3×10^{-3} - 103×10^{-3})**
Over Season Leaf (2)	V6 - V8	$67.3 \times 10^{-3} \pm 19.4 \times 10^{-3}$ (37.1×10^{-3} - 98.9×10^{-3})
Over Season Leaf (3)	V10 - V13	$44.6 \times 10^{-3} \pm 8.51 \times 10^{-3}$ (27.5×10^{-3} - 58.8×10^{-3})
Over Season Leaf (4)	V14 - R1	$56.9 \times 10^{-3} \pm 28.5 \times 10^{-3}$ (22.1×10^{-3} - 153×10^{-3})

Tissue Type	Crop Development Stage	Mean µg DvSnf7 dsRNA/g dw tissue ± SD (Range)
Over Season Root (1)	**V3 - V4**	**$23.9 \times 10^{-3} \pm 15.1 \times 10^{-3}$ (12.5×10^{-3} - 67.0×10^{-3})**
Over Season Root (2)	V6 - V8	$16.3 \times 10^{-3} \pm 4.84 \times 10^{-3}$ (6.62×10^{-3} - 25.7×10^{-3})
Over Season Root (3)	V10 - V13	$10.2 \times 10^{-3} \pm 4.77 \times 10^{-3}$ (5.13×10^{-3} - 24.3×10^{-3})
Over Season Root (4)	V14 - R1	$6.84 \times 10^{-3} \pm 2.67 \times 10^{-3}$ (2.66×10^{-3} - 13.0×10^{-3})
Over Season Whole Plant (1)	V3 - V4	$84.8 \times 10^{-3} \pm 43.8 \times 10^{-3}$ (51.1×10^{-3} - 213×10^{-3})
Over Season Whole Plant (2)	V6 - V8	$55.1 \times 10^{-3} \pm 23.1 \times 10^{-3}$ (33.0×10^{-3} - 106×10^{-3})
Over Season Whole Plant (3)	**V10 - V13**	**$25.5 \times 10^{-3} \pm 9.53 \times 10^{-3}$ (13.0×10^{-3} - 45.9×10^{-3})**
Over Season Whole Plant (4)	V14 - R1	$18.5 \times 10^{-3} \pm 6.27 \times 10^{-3}$ (10.3×10^{-3} - 32.2×10^{-3})
Forage Root	**R5**	**$2.37 \times 10^{-3} \pm 1.29 \times 10^{-3}$ (0.425×10^{-3} - 4.61×10^{-3})**
Forage	**R5**	**$4.26 \times 10^{-3} \pm 1.26 \times 10^{-3}$ (2.00×10^{-3} - 7.72×10^{-3})**
Senescent Root	R6	$1.39 \times 10^{-3} \pm 0.815 \times 10^{-3}$ (0.478×10^{-3} - 3.68×10^{-3})
Stover	R6	$0.677 \times 10^{-3} \pm 0.201 \times 10^{-3}$ (0.401×10^{-3} - 1.04×10^{-3})
Pollen	**VT-R1**	**$0.134 \times 10^{-3} \pm 0.090 \times 10^{-3}$ (0.073×10^{-3} - 0.292×10^{-3})**
Silk	R1	$5.42 \times 10^{-3} \pm 2.05 \times 10^{-3}$ (1.99×10^{-3} - 9.03×10^{-3})
Grain	**R6**	**$0.104 \times 10^{-3} \pm 0.033 \times 10^{-3}$ (0.056×10^{-3} - 0.175×10^{-3})**

[a] Crop development stages at which each tissue was collected; growth stages were described by Ritchie et al. (1997).
[b] Means, SDs, and ranges calculated from data collected at 5 sites (n=20, except senescent root n=19, stover n=16, pollen n=5, and grain n=18 due to expressions from two pollen samples that were <LOD and from the rest of the samples for senescent root, stover, pollen, and grain <LOQ).

2. Fate of DvSnf7 in the Terrestrial Environmental

The primary source of exposure of terrestrial nontarget organisms to the DvSnf7 expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn is expected to be the corn tissue. Movement of corn tissue influences the distribution and fate of DvSnf7 in the environment, since corn tissue will carry DvSnf7 to wherever it may move. How much DvSnf7 moves within the environment depends on what organ or tissue is moved and when, since expression levels differ between corn tissues, and change within them over the course of the growing season.

Prior to harvest, the majority of corn foliage expressing DvSnf7 will be contained within the planted field. Some breakage of foliage and other above ground plant parts may occur, which could result in their deposition outside field borders; however, movement of above-ground plant parts beyond the field border is expected to be minimal prior to harvest. Within soil, exposure is expected to be primarily limited to the roots, although sloughing of root cells into the surrounding soil will also occur. It is not known whether DvSnf7 would be present in root exudates, though upon root cell lysis, small amounts of DvSnf7 could be released into the surrounding soil.

During anthesis, corn pollen will shed and will be deposited on surfaces, including other plants, within the field and beyond the field borders. EPA has previously evaluated the potential for distribution of PIPs as a result of corn pollen deposition (USEPA 2010b). Corn pollen is of relatively large size among wind dispersed pollens (90 – 100 µm), which is thought to give it a greater tendency to settle out (Pleasants et al. 2001). In a study on milkweed plants in and near corn fields, Pleasants et al. (2001) showed that the majority of corn pollen stays within corn fields, and pollen levels follow an increasing trend with distance into the field (e.g., at one site, 147.5 grains/cm^2 were found on milkweed plants at 25 m into the field, whereas 55.5 grains/cm^2 were found 3 m into the field). In this study, mean pollen densities ranged up to 425.6 grains/cm^2 at 100% anthesis. Densities were also greater on milkweed plants growing within rows compared to those growing between rows, which shows higher deposition directly below the corn plants compared to immediate adjacent areas. Relatively small amounts of pollen dispersed beyond 5 meters from the field edge. Raynor et al. (1972) found that 63% of corn pollen remained within fields, 88% settled within eight meters of the field edge, and 98% settled within 60 meters. Only 0.2% of pollen was deposited at greater than 60 m from the corn field edge. Based on these data, density of corn pollen is understood to drop off very steeply within 10 – 15 meters from the edge of the field. These studies have shaped EPA's current understanding of PIP environmental fate resulting from pollen movement. More recent work has been done (e.g., see Gathman et al. 2006, Hoffman et al. 2014, Lang et al. 2015), and although sampling methods, sampling duration, and data analysis vary among all of these studies, they show similar deposition patterns, providing additional support for EPA's current understanding of this process as it affects PIP environmental fate.

After harvest, corn tissue may be left on the field, where it may remain or be subject to movement by wind and water. The amount and distance moved is not known and is expected to vary, but ultimately corn tissue that remains in the terrestrial environment is expected to become a part of the plant detritus upon and within soil. Additionally, corn plant material left on the field may be tilled into the soil. Eventually, cells of corn tissue will lyse and release into the soil any DvSnf7 that has not been broken down within the plant. Therefore, soil is expected to be the ultimate destination of DvSnf7 in the terrestrial environment.

Monsanto Company calculated an estimate of the maximum amount of DvSnf7 that could be present in soil, assuming all plants in a corn field are incorporated into the top 15 cm of soil. The estimate is based on an assumption of 9,000 lbs of corn plant residue at harvest (based on an assumption of 150 bushels per acre yield, as described in MRID 49315122), a maximum expression of 85 ng DvSnf7/g dw corn tissue (based on Over Season Whole Plant (1) value in Table 2), and soil bulk density of 1 g/cm^3 (based on soil bulk density ranging from 0.92 to 1.3 g

cm^3 for soils in MRID 49315122). These assumptions yielded an estimate of 0.571 ng DvSnf7/g soil. This estimate is expected to be high, since expression is much lower after harvest when most plant tissues would reach the soil environment. Nonetheless, using this estimate of corn plant weight per acre and the expression measured in stover and forage for MON 87411, as given in Table 2, concentrations in soil were calculated to be 0.00456 ng DvSnf7/g soil for stover, and 0.016 ng DvSnf7/g soil for forage.

Within soil, the DvSnf7 is expected to break down quickly. Monsanto Company submitted a laboratory study examining the aerobic degradation of the DvSnf7 dsRNA in soil (MRID 49315122). *In vitro* transcribed DvSnf7 test material (added at a rate of 7.5 µg DvSnf7/g soil) or a control substance was incorporated into soils collected from three different states in the U.S.: Illinois (IL), Missouri (MO) and North Dakota (ND). The DvSnf7 treatment also included MON 87411 leaf material (equivalent to adding 1.7 ng DvSnf7/g soil or 3 times the maximum amount of 0.571 ng DvSnf7/g soil calculated above). The soils differed in their physicochemical characteristics (silt, sand and clay composition, pH, organic matter, etc.) based on a pre-study analysis, and the USDA soil classifications were silt loam (IL), loamy sand (MO) and clay loam (ND). Soil samples were extracted and analyzed for DvSnf7 concentrations using QuantiGene® 2.0 kits obtained from Affymetrix, Inc. In addition, the decline in the insecticidal activity was quantified by using an insect bioassay with the Southern corn rootworm (*Diabrotica undecimpunctata howardi*; hereafter, SCR), which examined the mortality of insects exposed to extracts of the soil samples incorporated into their diet. The time required to reduce the DvSnf7 soil concentrations by 50% or 90% (DT_{50} and DT_{90} values, respectively) based on QuantiGene molecular analysis were the following: DT_{50} of 19 hr, 28 hr, and 15 hr and DT_{90} of 23 hr, 35 hr, and 22 hr for IL, MO, and ND, respectively. The decline in biological activity of DvSnf7 in soil, measured against standard concentration curves in the SCR bioassay, produced estimated DT_{50} and DT_{90} values of 18 hr, 29 hr, 14 hr and 23 hr, 34 hr, and 21 hr for IL, MO, and ND respectively. The bioassay data were also examined using a standard 3-parameter logistic equation to characterize the decline in insect mortality against incubation time, without conversion to soil concentrations. This approach produced DvSnf7 dissipation curves comparable to those obtained from the standard concentration analyses with QuantiGene and insect bioassay. In this evaluation, the estimated time to 50% dissipation of insect mortality was 23 hr, 33 hr, and 22 hr, and the 90% dissipation time was 55 hr, 45 hr, and 45 hr for IL, MO, and ND, respectively.

Results from the soil degradation study indicate that most of the DvSnf7 degraded within approximately 2 days after application to soil, regardless of texture, pH, clay content and other soil differences, as measured by molecular analysis (QuantiGene) and by insecticidal activity (bioassay). This study was initially submitted to support the MON 87411 seed increase registration, and originally EPA concluded that there appeared to be a low level of biological activity beyond the time that the dsRNA degraded below the quantification limits of the QuantiGene analysis. This conclusion resulted from the graphical presentations of the data in the study report, and Monsanto Company provided clarification to confirm that mortality observed after two days was within the range of control mortality during that phase of observations. Input of DvSnf7 into the soil is expected to occur to some degree throughout the growing season, and also afterward for as long as leaf tissue expressing or containing residues of DvSnf7 are present.

However, based on the results of this study, once it reaches the soil, it is unlikely to persist or accumulate.

3. Fate of DvSnf7 in the Aquatic Environment

As with terrestrial environments, movement of corn plant foliage beyond planted fields and into nearby aquatic habitats is expected to be limited prior to harvest. Pollen shed may deposit DvSnf7 into aquatic areas, though as described above, aquatic areas that are further than 10-15 m from the edge of a corn field are expected to receive minimal amounts of pollen expressing DvSnf7. DvSnf7 is also expressed at very low levels in MON 87411 and MON 89034 x TC1507 x MON 87411 x DAS-59122-7 pollen. Therefore, pollen from these corn plants is not likely a major contributor of DvSnf7 to aquatic exposure.

Post-harvest corn plant residue can enter nearby waterways, and may do so in large amounts in areas where corn is predominant within the landscape. Movement occurs by the action of wind and water (Griffiths et al. 2009, Tank et al. 2010) and inputs occur primarily in late fall and winter (Rosi-Marshall et al. 2007). All portions of corn plants that would be left over after harvest are typically observed in nearby aquatic areas, including leaves, stems, and cobs (Tank et al. 2010).

Much of what is understood about the environmental fate of PIP pesticidal substance comes from studies with *Bt* derived proteins (primarily Cry1Ab) in corn. With respect to aquatic nontarget exposure, EPA's position has been that concentrations of Cry proteins in water due to pollen shed have not been high enough to cause concern for potential adverse effects due to generally low levels of expression in pollen and low amounts of pollen entering nearby waterways. With respect to the potential exposure resulting from entry of corn detritus into aquatic systems, EPA's position has been the following:

"*...while postharvest crop residue was identified as the most likely route of exposure (Carstens et al. [2012]), aquatic exposure to biotech crops has been shown to be limited temporally and spatially with low to negligible exposure concentrations of Cry proteins in post-harvest crop tissues (Swan et al. 2009, Chambers et al. 2010, Jensen et al. 2010, Wolt and Peterson 2010, Carstens et al. [2012])*" (USEPA 2010a, 2010b).

These conclusions were initially made in 2010, and in light of them, EPA has assumed that exposure in aquatic environments resulting from entry of corn plant debris into water is low, and has thus not required studies on aquatic species to support *Bt* PIP registrations. EPA's general understanding based on research on this topic is that corn plant debris does not enter nearby waterways in any predictable pattern, and once it does, it is broken down somewhat rapidly first by microorganisms and physical means, and later by invertebrate consumption (Griffiths et al. 2009, Tank et al. 2010). Corn tissue must break down somewhat in the water for one to two weeks before it is suitable for consumption by invertebrates (Chambers et al. 2010, Jensen et al. 2010). During this time, leaching of the Cry protein from leaf tissue is rapid, though it is not necessarily complete (Chambers et al. 2010, Böttger et al. 2015, Strain and Lydy 2015). Therefore, Cry proteins are not considered to be present in corn detritus in significant amounts by the time aquatic invertebrates would be able to consume it (after approximately two weeks). Corn leaf detritus takes approximately 60-70 days to completely break down in water (Wolt and

Peterson 2010, Tank et al. 2010). Other plant parts such as cobs and stalks appear to take longer to degrade (Chambers et al. 2010, Tank et al. 2010); however, it is unclear whether they represent the same potential source of exposure, since they become clumped and accumulate locally in large amounts with the stream. Chambers et al. (2010) noted that this type of plant material was uncommon in benthic samples. Once in water, Cry proteins break down rapidly, but breakdown may be slowed by cold temperatures (Strain and Lydy 2015). It is noted that several of the cited field studies involving tissue and Cry protein breakdown took place during winter months.

What is evident from the above research is that exposure of aquatic organisms to Cry proteins as a result of consumption of corn plant debris is unlikely to be significant, though Cry proteins are apparently present in water to some degree for a short while after corn tissue enters water. Strain and Lydy (2015) showed the presence of Cry1Ab in runoff; however, given the amount of debris that can enter waters adjacent to corn fields, leaching from debris in the water may be a bigger contributor to the presence of Cry proteins in the water column. Several studies above indicate that the majority of leaching would occur within two weeks of entry into water, after which time exposure would be expected to decrease; however, entry of corn detritus into nearby aquatic systems is not necessarily a one-time event, and may occur several times between harvest and planting the following year. This understanding is relevant to the fate of dsRNA in water, since it is highly hydrophilic. Therefore, dsRNA likely has a tendency to follow a similar pattern of leaching from corn plant debris, so aquatic exposure assumptions for Cry proteins would also apply to DvSnf7.

The degradation of DvSnf7 in soil is expected to limit its presence in runoff. As noted above, DvSnf7 largely degraded within 2 days in soil, which is likely due to the presence of RNA hydrolyzing bacteria and RNases that are present there. It is not known whether the environmental fate profile of all dsRNA is similar to DNA; however, preliminary data show that DvSnf7 has a similar pattern of degradation in water as DNA (see below). If that is the case, then there is some evidence that interaction with the soil would assist its breakdown in leachate water moving through soil. For example, Gulden and colleagues (2005) measured half-lives ($DT_{50}s$) of recombinant DNA from transgenic plants in leachate water, which ranged from 1.2 to 26.7 hours. In a review on this subject, Nielsen et al. (2007) report both similar and longer times for detection of DNA in water, though they also note limitations of quantitative analyses for DNA in the environment.

Nonetheless, similar to the analysis performed for soil, a worst-case estimate may be calculated for the concentration of DvSnf7 in water. Consistent with previous analyses (USEPA 2010b) a standard field-pond scenario (wherein a 1 ha pond, 2 m deep, draining a 10 ha area planted with corn is assumed) was used to calculate exposure. Assuming 9,000 lbs corn tissue/acre and expression levels for stover and forage as assumed for soil, concentrations of DvSnf7 in water would be estimated as 0.0014 ng DvSnf7/mL water assuming the expression level for stover and 0.0087 ng DvSnf7/mL water assuming the expression level for forage.

The deficiency letter issued during the review for the MON 87411 seed increase registration, noted that, should EPA determine that DvSnf7 persists in leaf material post-harvest, additional testing with aquatic organisms would be required. In response, Monsanto Company submitted

rationale to justify that DvSnf7 would be expected to degrade quickly in aquatic systems. This rationale included reference to preliminary data showing that DvSnf7 degrades in sediment and water. These data have recently been submitted, but are still considered preliminary, since they are contained within a submitted manuscript for publication, which has been accepted by the journal with revisions at the time of this writing (Fischer et al., in press). In this study, degradation kinetics of DvSnf7 (added at a rate of 300 – 330 ng DsRNA/mL water) were observed in sediment and water collected from a lentic (with loamy sand) and a lotic (with clay loam) system. DT_{50} and DT_{90} values were calculated for water and sediment from disturbed and undisturbed sediment-water microcosms and in filtered water or sediment alone. Degradation was determined with both molecular analyses and bioassays examining SCR mortality. Across all treatments, half-lives of DvSnf7 in water from undisturbed sediments were < 3 d, with DT_{90} < 5 d whereas in water from disturbed sediment treatments half-lives were < 1 d. DvSnf7 was inconsistently detected at low levels in undisturbed sediment overlaid by water. In disturbed sediments, degradation kinetics could only be determined in the clay loam sediment (DT_{50} = 2.6 d), due to low levels of detection in the loamy sand sediment, and in all samples DvSnf7 was undetectable within 4 to 6 d. In sediments treated directly (no overlaying water), half-lives were similar to those observed in other sediment samples, but DvSnf7 was detectable for longer periods, ranging between 14 and > 28 days. The results suggest that degradation of DvSnf7 occurs predominantly in water, where it dissipates quickly. The influence of clay binding was evident in the disturbed sediment and sediment-only treatments; however, dissipation was still rapid in these samples, with no detectable biologically active DvSnf7 after 14 days.

These results are limited somewhat in that they involve test sediment and water from only two locations. Testing using a sediment and water collected across a broader range of locations representing corn growing areas would yield more generalizable results. However, the results in this study are consistent with those of other studies examining degradation of DNA or RNA in water. For example, Zhu (2006) observed plasmid and plant DNA to be completely or mostly degraded in river and ground water by 96 h. In lake water, DNA and RNA hydrolyzing bacteria degraded DNA and RNA to undetectable levels by 10 and 8 days, respectively (Takata et al. 1993). Eichmiller et al. (2016) observed rapid degradation of environmental DNA in water between 3-8 days, followed by a slower rate of decay, and degradation was influenced by water quality and temperature (the slowest decay occurred with DT_{90} = 6.6 d at 5°C). Additionally, it is noted that the study from Monsanto Company was performed with sediment-water samples spiked with naked dsRNA, as opposed to samples treated with the PIP plant material. The dsRNA in the above study would not be associated with cell wall fragments, proteins, or other cellular metabolites, which would be present in water containing decaying PIP plant material and would likely also serve to degrade dsRNA. Therefore, the degradation estimates may be high compared to those that would occur in natural environments.

D. Nontarget Effects Data

Two separate SAP reports (FIFRA SAP 2001, 2002) have previously recommended that nontarget testing for PIPs (in those cases, *Bt* derived proteins) should focus on invertebrate species exposed to the crop in which the PIP(s) will be expressed. EPA has previously determined, based on these recommendations, that non-target organisms with the greatest exposure potential to PIP pesticidal substances in transgenic corn fields are beneficial insects that

feed on plant tissues (e.g., pollen), so terrestrial invertebrates are the focus of nontarget organism testing and of PIP risk assessments. Nontarget insects were also the focus of the recommendations of the 2014 SAP regarding unintended effects of dsRNA-based pesticides (FIFRA SAP 2014). Nonetheless, EPA has recognized that exposure to other nontarget organisms can occur (e.g., exposure to birds by consumption of grain or pest insects that have consumed the PIP), and has required testing on representative species.

In the absence of PIP-specific data requirements, EPA has historically required applicants for PIP registrations to meet the 40 CFR Part 158.2150 data requirements for microbial pesticides. These requirements include testing on birds, mammals, nontarget insects, honey bee, plants, and aquatic animal species. The October 2000 SAP recommended that while actual plant material is the preferred test material, bacteria-derived active ingredient is also a valid test substance, particularly in scenarios where test animals do not normally consume corn plant tissue and where large amounts of active ingredient are needed for maximum hazard dose testing (FIFRA SAP 2001). These recommendations were made at the time for *Bt*-derived PIPs, but similar principles would also apply to dsRNA-based PIPs. Test substances used in studies submitted to support the MON 87411 seed increase registration and the proposed registration for MON 89034 x TC1507 x MON 87411 x DAS-59122-7 included *in vitro* transcribed DvSnf7 dsRNA test material as well as plant material (grain) from MON 87411 corn. Comparative analyses showed the equivalence of the *in vitro* transcribed and purified DvSnf7 dsRNA test material with the active ingredient expressed in MON 87411 corn (USEPA 2015).

Although it is recommended for non-target testing to be conducted at a test dose at least 10 times the EEC whenever possible, BPPD has accepted test dose margins less than 10 times for cases in which the uncertainty in the system is low or where high concentrations of test material are not possible to achieve. BPPD has also allowed for testing at lower doses in cases where many species are tested or tests are very sensitive, although the concentration used must exceed the EEC.

Maximum hazard dose testing on representative organisms from several taxa was performed with DvSnf7 in support of the proposed registration of MON 89034 x TC1507 x MON 87411 x DAS-59122-7. Several of these studies were submitted to support the MON 87411 seed increase registration, and many of them were determined at the time to be supplemental due to a need to clarify certain aspects of study conduct and results. Additional data have been provided since the 2015 ecological risk assessment for MON 87411, and updated study summaries are presented where applicable. Additional data to support a full commercial registration involving DvSnf7 was also required by EPA and submitted subsequent to the 2015 ecological risk assessment. These studies are also summarized below. In addition to effects testing, a study to determine soil degradation of DvSnf7 has also been submitted (discussed above), as well as a bioinformatic study and a field study on effects to nontarget arthropods in agricultural environments. Rationales justifying why testing is not required were also submitted to address certain data requirements. The individual results for nontarget organism and soil degradation testing for DvSnf7 dsRNA are summarized in Table 3. The studies are described in more detail below, and full reviews of each study can be found in the individual Data Evaluation Records.

Table 3. Summary of data submitted for DvSnf7 dsRNA to comply with data requirements published in 40 CFR § 158.2150.

Data Requirement	OCSPP Guideline	Test Substance	Results Summary and Classification	MRID No.
Avian dietary testing, broiler chicken, *Gallus domesticus*	885.4050	MON 87411 corn grain	A 42-day dietary study showed no adverse effects on survival, broiler performance, or carcass yield in broiler chickens fed a diet containing 57% MON 87411 grain. **Classification: Supplemental**	49315111
Avian dietary testing, Northern bobwhite (*Colinus virginianus*)	850.2100	*In vitro* transcribed DvSnf7	A dietary study showed no adverse effects on survival or body weight gain of Northern bobwhite with a 14-day exposure to DvSnf7 at 1000 µg/kg diet. **Classification: Acceptable**	49886501
Avian inhalation testing	885.4100	N/A	Not required. Inhalation is not expected to be a route by which birds may be exposed to DvSnf7 dsRNA.	N/A
Wild mammal testing	885.4150	N/A	Studies with laboratory animals and additional rationale are sufficient to determine risk to wild mammals. Adverse effects were not observed in a 28-day mouse study with purified DvSnf7 dsRNA or in a 90-day rat study with MON 87411 corn grain included in the diet at 33%. **Classification: Acceptable**	49505804 49505806
Freshwater fish testing, channel catfish, *Ictalurus punctatus*	885.4200	MON 87411 corn grain	In an 8-week study, consumption of MON 87411 corn grain at the exposure level tested (30% of the diet) had no adverse effects on survival, weight gain, or diet conversion. Additional rationale also supported this data requirement (see freshwater invertebrate testing below) **Classification: Supplemental**	49505805 49553302
Freshwater invertebrate testing	885.4240	N/A	Submitted rationale provided justification for not testing freshwater invertebrates. Exposure to DvSnf7 is expected to be low and it is not expected to persist in aquatic environments. **Classification: Supplemental**	49553302
Estuarine and marine animal testing	885.4280	N/A	Data were not required, since significant exposure is not expected in these environments.	N/A
Non-target plant testing	885.4300	N/A	Not required. Exposure to nontarget plants is expected to be minimal.	N/A
Non-target insect testing, lady beetle, *Coleomegilla maculata*	885.4340	*In vitro* transcribed DvSnf7	No adverse effects on survival, development to adult stage, time to emergence, or adult biomass were observed in *C. maculata* fed 1000 ng DvSnf7 dsRNA/g diet for 21 days. **Classification: Acceptable**	49315114
Non-target insect testing, parasitic wasp, *Pediobus foveolatus*	885.4340	*In vitro* transcribed DvSnf7	No adverse effects were observed on adult survival in *P. foveolatus* fed a 30% honey/water solution containing 1000 ng DvSnf7 dsRNA/g diet in a 20-day study. **Classification: Acceptable**	49315115
Non-target insect testing, insidious flower bug,	885.4340	*In vitro* transcribed DvSnf7	No adverse effects were observed on survival or rate of adult emergence in *O. insidiosus* nymphs	49315117

Data Requirement	OCSPP Guideline	Test Substance	Results Summary and Classification	MRID No.
Orius insidiosus			fed 1000 ng DvSnf7 dsRNA/g diet in a 10-day study. **Classification: Acceptable**	
Non-target insect testing, carabid beetle, *Poecilus chalcites*	885.4340	*In vitro* transcribed DvSnf7	No adverse effects on survival, development to adult stage, time to emergence, or adult biomass were observed in carabid beetle larvae fed 1000 ng DvSnf7 dsRNA/g diet in a 35-day study. **Classification: Acceptable**	49315119
Non-target insect testing, green lacewing, *Chrysoperla carnea*	885.4340	*In vitro* transcribed DvSnf7	No adverse effects on survival or reproduction were observed in *C. carnea* exposed to a nominal concentration of 1001 ng DvSnf7 dsRNA/g diet in an 18-day study. **Classification: Acceptable**	49886502
Non-target insect testing, rove beetle, *Aleochara bilineata*	885.4380	*In vitro* transcribed DvSnf7	No adverse effects were observed on reproduction in beetles exposed to 1000 ng DvSnf7/g diet in a 28-day study. **Classification: Acceptable**	49886503
Honeybee testing, adults *Apis mellifera*	885.4380	*In vitro* transcribed DvSnf7	No adverse effects on survival were observed when honey bee adults fed on a sucrose solution containing 1000 ng DvSnf7/g diet for 14 days. **Classification: Acceptable**	49315113
Honeybee testing, larvae *Apis mellifera*	885.4380	*In vitro* transcribed DvSnf7	No adverse effects were observed in survival or development of larvae provided a single dose equivalent to 11.3 ng DvSnf7 dsRNA per bee in a 14-day study. **Classification: Acceptable**	49315112
Earthworm toxicity, *Eisenia andrei*	850.6200	*In vitro* transcribed DvSnf7	No adverse effects on survival or percent change in biomass in earthworms exposed to 5000 ng DvSnf7 dsRNA/g artificial soil in a 14-day contact study. It is unclear whether this route of exposure is relevant, and uncertain whether the DvSnf7 was present in the test matrix for the entire duration of the study. **Classification: Supplemental**	49315116
Non-target insect (soil arthropod) testing, springtail, *Folsomia candida*	885.4340	*In vitro* transcribed DvSnf7	No adverse effects on survival or reproduction were observed in *F. candida* exposed to 1000 ng DvSnf7 dsRNA/g diet in a 28-day study. **Classification: Acceptable**	49315118
Soil fate and degradation	885.5200	*In vitro* transcribed DvSnf7 plus MON 87411 plant tissue	Based on Quantigene® 2.0 analysis of soil and bioactivity measured in Southern corn rootworm, DvSnf7 dsRNA degrades rapidly in soil with 90% dissipation time ranging 45 - 55 hr. **Classification: Acceptable**	49315122
Field evaluation of nontarget arthropod abundance	850.2500	MON 87411 corn plants	No evidence of adverse effects to nontarget arthropod populations or communities were observed between MON 87411 corn plots and genetically similar (except for transgenic traits) controls in field plots studied in the U.S., Brazil, and Argentina. The data appear to show no effects; however, the study design introduces too much uncertainty to definitively determine effects among treatments.	49553304

Data Requirement	OCSPP Guideline	Test Substance	Results Summary and Classification	MRID No.
			Classification: Supplemental	
Nontarget organism bioinformatic analysis	N/A	DvSnf7 Sequence	No exact ≥ 21 nt matches were found when the 240 nt DvSnf7 sense strand sequence was queried against transcriptome sequence collections from 23 nontarget organisms. **Classification: Supplemental**	49553306

E. Study Summaries for DvSnf7 dsRNA

1. Birds

Avian Dietary Toxicity

Broiler Chicken

A 42-day feeding assessment using Cobb × Cobb 500 broilers (*Gallus domesticus*) was conducted to compare the nutritional value of diets containing MON 87411 maize grain and a near isogenic conventional maize control NL6169 with similar background genetics to MON 87411 (MRID 49315111). This study was originally submitted to support the seed increase registration of MON 87411. Five additional diets containing maize grain representatives of the population of commercial conventional maize varieties were included. Nutrient analyses of each maize grain source were used to formulate diets having identical nutrient specifications, which were fed to the broilers *ad libitum* throughout the study. Treatments were assigned to pens randomly within five blocks of 14 pens each (seven male and seven female) with ten broilers per pen for a total of 70 pens and 700 birds. Broilers were weighed on Day 0 and at the end of the study just prior to processing to assess carcass yield. Pen feed intake was determined for the duration of the study, and used to calculate feed to weight gain ratio.

There were no biologically relevant differences in broiler performance, which included observations of average weight, average weight gain, feed intake, and feed:weight gain ratio; carcass yield, which included carcass weight and weights of various carcass parts; or mortality (mortality ranged from 0-0.8% across all treatments) between broilers fed diets containing MON 87411 maize grain and those fed diets containing near isogenic conventional control NL6169 maize grain. The study report does not state the percentage of grain in the diet; however, an environmental risk assessment submitted by Monsanto Company (MRID 49505802) states that the percentage of MON 87411 in the diet was 57%. While this is more than half of the diet, expression of DvSnf7 dsRNA in MON 87411 grain (0.104×10^{-3} µg/g tissue dwt) and MON 89034 x TC1507 x MON 87411 x DAS-59122-7 grain (0.089×10^{-3} µg/g tissue dwt) is much lower than in other plant tissues. This level of exposure is not a maximum hazard dose or concentration that is typically required for Tier I nontarget organism studies, and it is also unclear whether this amount is representative of environmental concentrations, since birds may be exposed through routes other than consumption of corn grain (e.g., consumption of pest insects that have consumed leaf or other tissue). Also, while the other variables measured (e.g., weights of various body parts) may indicate the nutritional performance of the grain, it is unclear

what relevance they have to survival or reproduction of wild birds. While this study does provide some insight into the potential for adverse effects in birds, particularly for extended exposure periods (6 weeks), its usefulness in determining risk depends on the actual level of exposure to birds in the field. Because of this limitation, the study was determined to be supplemental.

Northern Bobwhite

Due to the limitations described for the study with broiler chickens, EPA required additional guideline testing with birds in the deficiency letter issued during the review for the MON 87411 seed increase registration. Rationale was submitted in response, which was determined to be sufficient for the limited, seed-increase registration; however, this study was required for the full commercial registration. The required testing was to be performed at a higher concentration to account for exposures in the field that may be higher, and was also required to be extended in duration to ensure that latent effects that may result from gene silencing would be observed.

To address this requirement, Monsanto Company conducted an additional dietary toxicity study with the Northern bobwhite (*Colinus virginianus*; MRID 49886501). The study was conducted according to the OCSPP 850.2100 guideline, with some modifications that included extending the exposure and observations period to 14 days. The study involved a single test concentration (nominal: 1000 µg DvSnf7 per kg of diet) and a control group (deionized water). The diet used in the study was a commercial game bird ration, and test diets were fed *ad libitum* for the duration of the 14-day study. Each test group consisted of 30 birds (six pens per treatment, each pen containing five birds), and birds were 14 days old at study initiation. All birds were observed at least twice daily throughout the study for signs of toxicity and abnormal behaviors, and survival and body weight (measured on individuals on Days 0, 7, and 14) were evaluated. Feed consumption was determined for each pen at approximately 24-hour intervals from test initiation to test termination on Day 14.

After 14 days of continuous dietary exposure to the DvSnf7 dsRNA at 1000 µg DvSnf7/kg diet, no adverse effects on survival or weight gain were observed in the Northern bobwhite. Survival was 100% in both test groups, and both groups gained 43 g body weight over the course of the study. No overt signs of toxicity (e.g. convulsions, loss of coordination, etc.) or abnormal behavior (e.g. hyperexcitability, lethargy, etc) were observed in the control or test substance treatments, and food consumption did not differ significantly. Based on the results, the 14-day dietary LC_{50} for Northern bobwhite exposed to DvSnf7 was greater than a nominal concentration of 1000 µg DvSnf7/kg diet.

The study generally followed the guideline described, with the exception of the concentration tested. The concentration suggested for a limit test in the OCSPP 850.2100 guideline is 5000 ppm, whereas this study tested 1000 ppb or 1 ppm. Given that DvSnf7 is expressed at very low levels in MON 87411 (e.g., highest mean dry weight expression level for any tissue was 97×10^{-3} µg/g in leaf [U.S. trials], and for grain was 0.104×10^{-3} µg/g dry weight [Argentina trials]) and MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn tissue (e.g., highest mean dry weight expression is 89.0×10^{-3} µg/g in leaves and 0.089×10^{-3} µg/g dry weight for grain), the nominal amount tested was 10.3 times the highest mean dry weight expression level measured

for MON 87411, and 11.2 times the highest mean dry weight expression in MON 89034 x TC1507 x MON 87411 x DAS-59122-7. Compared to expression in grain, the level tested is >9,500 times the level expressed in MON 87411 and is > 11,000 times the level expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 grain. Therefore, while this study did not expose the birds to the recommended limit dose of 5000 ppm, it is understood that the test was performed at an exposure level many times higher than that expected of avian food sources in the environment for these corn plants. This approach is consistent with the maximum hazard concentration approach that is utilized for biopesticides, and the study was classified as acceptable.

Avian Inhalation Toxicity

Avian inhalation toxicity is a conditional data requirement of 40 CFR 158.2150. EPA notes that corn pollen was determined not to be respirable in the human health assessment for DvSnf7 (USEPA 2016). Significant exposure of birds to DvSnf7 dsRNA via inhalation is not anticipated, since the primary route of exposure is considered to be ingestion of plant material expressing DvSnf7. Therefore, these data are not required.

2. Wild Mammals

Two studies are available with which to assess the potential toxicity of DvSnf7 in wild mammals, including a 28-day acute oral toxicity study with mice (MRIDs 49505804 and 49576101) and a 90-day feeding study with rats (MRIDs 49505806 and 49576101). These studies were reviewed by EPA and classified as acceptable (U.S EPA 2015). In the 28-day study, mice were dosed daily with DvSnf7 test material with purity of 100% a.i., and no adverse effects were observed that were attributable to the test material. This study established a NOAEL of 105 mg/kg/day. In the 90-day study, rats were fed a diet of 33% grain, and no treatment-related effects were observed on mortality, clinical signs, functional observational battery, body weight, body weight gain, food consumption, food efficiency, clinical pathology, organ weights, gross pathology, or microscopic pathology. Based on information described by Monsanto Company, the NOAEL was determined to be 1899 and 2303 mg/kg total cage body weight per day for males and females, respectively; however, these values appear to describe the NOAEL for MON 87411 grain in the diet, and not the NOAEL for DvSnf7 dsRNA.

3. Freshwater Fish and Invertebrates

Freshwater Fish Toxicity

Channel Catfish

A dietary bioassay with channel catfish (*Ictalurus punctatus*) was conducted to address the Tier I data requirement for freshwater fish. This study was originally submitted to support the seed increase registration of MON 87411, and no additional information was required or submitted to upgrade its status. This study was designed to evaluate the nutritional equivalence of diet containing maize grain from MON 87411 compared to diets containing maize grain from a near isogenic conventional control or from each of four reference maize varieties. The diets were

formulated to contain 30% of the appropriate maize grain variety, to contain approximately 32 ± 1% crude protein, and to be isonitrogenous. Groups of 100 fish each (five replicates per group) were fed the appropriate diet daily for eight weeks. After eight weeks, there were no statistically significant differences among the treatment groups for food consumption, weight gain, or diet conversion, and grain from MON 87411 corn was considered to be nutritionally equivalent to conventional maize grains when fed to channel catfish. While this information is helpful, this study has similar limitations for determining toxicity in freshwater fish as described for the broiler chicken study above. In this case, the diet consisted of just 30% of grain, in which relatively low levels of DvSnf7 are expressed compared to other corn tissues. The study shows that adverse effects are not likely based on the study conditions, and this information is useful to the risk assessment. However, the certainty of risk estimated from this study is dependent on the actual exposure in the field. For this reason, the study was elassified as supplemental.

Additional Rationale

Justification for no additional testing, including chronic testing with freshwater fish, was given in rationale submitted as a response to a deficiency letter issued prior to the MON 87411 seed increase registration (MRID 49553302). The deficiency letter stated that if EPA determined the DvSnf7 was likely to persist in corn plant tissue, thus increasing tbe potential for exposure in freshwater environments, then additional aquatic testing would be required. Such testing would include a 21-day *Daphnia* study conducted according to OCSPP guideline 885.4240 or a similar study involving a freshwater invertebrate shredder species (e.g., see Carstens et al. 2012), and a freshwater fish lifecycle bioassay conducted according to OCSPP guideline 850.1500. The requirement also included a freshwater fish toxicity study conducted according to OCSPP guideline 885.4200; however, the above study had been submitted independently of the response to the deficiency letter.

To justify the lack of persistence in corn tissue, the rationale discussed the lack of persistence of Cry proteins in field studies to show that corn plant tissue does not provide a protective structure leading to persistence of these proteins, which would also be true for DvSnf7. Additional justification included results of the soil degradation study and preliminary results of the degradation study in water and sediment, which are both discussed above, as well as studies from open literature indicating short persistence time of DNA in soil and water. Monsanto Company also made the point that EPA had previously determined that exposure of Cry proteins to aquatic organisms is low, and that determination would apply also to DvSnf7. Further, they argued that DvSnf7 is highly specific for its target gene in WCR, barriers to dsRNA uptake exist for both invertebrates and vertebrates that reduce bioavailability, and that a bioinformatic analysis (discussed below) returned no matches between the DvSnf7 240nt dsRNA and transcriptomes of freshwater vertebrates and invertebrates included in the search.

EPA classified the rationale as supplemental, based on indications that exposure to DvSnf7 as expressed in the MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn in aquatic environments is expected to be low and is not expected to persist (see discussion in Section F below). EPA may ask for additional testing, as previously indicated, if new information becomes available or this understanding of exposure changes.

Freshwater Invertebrate Toxicity

This data requirement was addressed with the same rationale as described above, and was determined to be supplemental for the same reasons.

4. Marine and Estuarine Fish and Invertebrates

As with freshwater environments, EPA anticipates that exposure levels to DvSnf7 in marine and estuarine environments will be low, and that DvSnf7 will not persist in water. Data have not been required to address toxicity to marine/estuarine fish and invertebrates for this proposed registration; however, additional data will be required if information is received to cause these exposure estimates to change.

5. Nontarget Plants

Data were not required because significant exposure to plants is not anticipated. More discussion is provided in Section F below.

6. Nontarget Insects and Other Invertebrates

Nontarget Insects

Lady Beetle

Larvae of the lady beetle, *Coleomegilla maculata* (Coleoptera: Cocinellidae), were tested in a 21-day study to determine the potential toxicity of DvSnf7 to this nontarget insect (MRID 49315114). Larvae were exposed to DvSnf7 in a test diet at a nominal concentration of 1000 ng/g of diet. A negative water control and a positive control of potassium arsenate were also tested. Exposure to *C. maculata* to the three diets was replicated three times with 20 individually housed insects per diet replicate for a total of 60 insects per diet treatment. All dietary exposures were initiated with the first instar larvae, and the study was continued until all surviving control and test insects developed to the adult stage. Survival, development to adult stage, development time (days) to adult emergence, and adult biomass were observed. Mean percent survival at test termination for the DvSnf7, negative control, and positive control diets were 92%, 90%, and 17%, respectively. None of the larvae fed the positive control diet developed to the pupa stage. The mean percent development to adult for larvae exposed to the DvSnf7 and negative control diets were 92% and 90%, respectively, and development time to emergence for both treatment groups was 15 days. Mean adult biomass was 10.2 mg for both the DvSnf7 and control treatments. The study authors reported a NOEC of $\geq$ 1000 ng DvSnf7/g diet. The results of this study show that exposure to DvSnf7 at the maximum hazard concentration of 1000 ng/g of diet had no adverse effect on the survival, development, and growth of this species.

This study was originally submitted in support of the seed increase registration for MON 87411, and was determined to be supplemental, but upgradeable, if raw data for the observations made in the study and food consumption data or other information confirming equal food consumption

were provided. Sufficient data to address these deficiencies were provided, and the study was upgraded to acceptable.

Parasitic Wasp

Toxicity of DvSnf7 was tested with the parasitic wasp (*Pediobius foveolatus*) in a 20-day study. *P. foveolatus* were exposed to DvSnf7 test material at 1000 ng/g in a 30% honey/water (v/v) solution (MRID 49315115). An assay control treatment of 30% honey/water solution and a positive control diet at 200 µg/g potassium arsenate in a 30% honey/water solution were also included. Four replicates of 10 adult wasps per replicate were included for a total of 40 wasps per treatment. All dietary exposures were initiated with newly emerged adults after approximately 24 hours of acclimation, and feeding *ad libitum* was allowed for the duration of the study. There was no mortality of *P. foveolatus* adults that had fed the DvSnf7 treatment diet or the assay control diet after 20 days. In contrast, the *P. foveolatus* adults fed the potassium arsenate treatment diet showed 100% mortality at day 12. The study authors reported a NOEC of ≥ 1000 ng DvSnf7/g diet. The results of this study show that continuous dietary exposure to DvSnf7 for 20 days at a concentration of 1000 ng/g diet had no effect on the survival of the parasitic wasp, *P. foveolatus*.

This study was submitted to support the seed increase registration for MON 87411, and was originally determined to be supplemental, but upgradeable, since confirmatory information was missing. Additional information required included an explanation for a discrepancy in a sub-report describing samples tested for dose confirmation and test material stability, clarification on units used in calculating the nominal dose, and information confirming consumption of the test diets. Clarifying information was provided, and the study was reclassified as acceptable.

Insidious Flower Bug

Toxicity of DvSnf7 to insidious flower bug (*Orius insidiosus*) nymphs was determined in a study involving 10 days of exposure (MRID 49315117). The insects were allowed to feed *ad libitum* on a diet containing DvSnf7 at 1000 ng/g diet. A positive control diet containing 100 µg of potassium arsenate per gram diet and a negative control diet were also included. Each treatment consisted of 40 individually housed nymphs, which were initially exposed as 5-day old nymphs. Survival and development were observed. Survival of the test nymphs was 93% in both assay control and DvSnf7 treatment. Development to adult occurred at 95% and 98% in the assay control and DvSnf7 treatment, respectively. The average time for test nymphs to develop into adults was 11.11 ± 0.15 days in the assay control treatment and 10.87 ± 0.13 days in the DvSnf7 RNA treatment. In contrast the *O. insidiosus* nymphs fed the potassium arsenate diet showed 100% mortality by day 10 with 13% of nymphs developing to adults. The study authors reported a NOEC of ≥ 1000 ng DvSnf7/g diet. The results of this study show that continuous dietary exposure to DvSnf7 for 10 days at a concentration of 1000 ng/g diet had no effect on the survival and development of *O. insidious* nymphs.

This study was originally submitted to support the seed increase registration for MON 87411, and was previously classified as supplemental, hut upgradeable if information was provided to clarify the length of time that observations were made beyond the 10-day exposure, consumption

of food containing test material, and if raw data were provided. This information was provided and the study was upgraded to acceptable.

Carabid Beetle

In a 35-day dietary toxicity study, carabid beetles (*Poecilus chalcites* [Say]) were exposed to DvSnf7 test material at a nominal concentration of 1,000 ng/g of diet (MRID 49315119). A water assay control and a potassium arsenate positive control were also used. Exposure of the carabid beetles to the three diets was replicated three times with 20 insects per diet for a total of 60 insects per diet treatment. All dietary exposures were initiated with the first instar larvae. Survival, development to adult stage, developmental time (days) to adult emergence, and adult biomass were measured. Mean percent survival at test termination for the test, control, and positive control diets were 93%, 92%, and 65% respectively. None of the larvae fed the positive control diet developed to the pupa stage. The lower survival and inhibited development of insects fed potassium arsenate confirmed that the test system was capable of detecting toxic effects through the dietary exposure; however, in most other studies test insects did not survive the positive control treatment. The percent development to adult for larvae exposed separately to test and assay control diets averaged 70% and 75%. For the test and assay control diet treatment groups, the development time to adult emergence averaged 33 days. Adults that were fed test and control diet treatments had mean biomass of 31.9 mg and 32.3 mg, respectively. The study authors reported a NOEC of $\geq$ 1000 ng DvSnf7/g diet. The results of this study show that exposure to DvSnf7 at the maximum hazard dose concentration of 1000 ng/g diet had no adverse effect on the survival, development, and growth of the carabid beetle, *Poecilus chalcites*.

This study was originally submitted to support the seed increase registration of MON 87411, and was classified as supplemental, but could be upgraded if clarification was provided regarding the typical mortality of *P. chalcites* exposed to potassium arsenate or explanation was provided of the relatively high survival in this group. Additionally, confirmation of the food consumption, and raw data on cumulative mortality, development time, and on adult biomass were requested. This information was provided, and was sufficient to upgrade the classification of the study to acceptable.

The EPA dsRNA white paper (USEPA 2013) discussed potential unintended effects of dsRNA-based pesticides, such as immune stimulation, over-saturation of RNAi machinery, and off-target effects. Off-target effects may result from binding of siRNAs to genes other than those with the target sequence (Birmingham et al. 2006, Jackson et al. 2003), which could result is unpredicted downregulation of genes within the genomes of nontarget organisms. Due to uncertainties regarding these potential unintended effects related to DvSnf7 exposure, EPA required two additional studies with nontarget insects that specifically included reproductive endpoints for a full commercial registration involving MON 87411. Only one study was available for the seed increase registration (see below). Two additional studies were submitted, involving green lacewing (*Chrysoperla carnea* Steph. [Neuroptera: Chrysopidae]) and rove beetle *Aleochara bilineata* (Coleoptera, Staphylinidae).

Green Lacewing

The purpose of this study was to evaluate the potential effect of a chronic dietary exposure (up to 18 days) to DvSnf7 on the survival and reproductive performance (i.e., egg production and egg viability) of adults of the green lacewing, under laboratory test conditions (MRID 49885602). Testing was performed according to the guideline described by Vogt et al. (2000). Newly-emerged adult (< 24 h old) lacewings were fed *ad libitum* an artificial diet medium into which individual treatments had been incorporated. The test item was evaluated at a single concentration, equivalent to 1001 ng DvSnf7/g diet. The other treatments evaluated were a distilled water control and a toxic reference of teflubenzuron (0.01 mg a.i./g diet). Each treatment included three replicates of green lacewing insects, each with 10 males and 10 females, which were provided with treated diet. Once the insects had matured sexually, observations were made for any sub-lethal effects on their reproductive performance (i.e. egg production and egg viability). These assessments were carried out 14-18 days after the bioassay commenced for the DvSnf7 and toxic reference treatments, and 15-18 days after commencement for the control treatment (approximately one week after eggs were observed in all of the individual boxes of insects). The egg-laying activity was monitored for two 24-h periods and the viability (percentage hatch) of the eggs they produced was also recorded.

In the control and test item treatments, the mean numbers of eggs produced per female per day were 20.4 and 21.8, respectively, and mean percent egg viability was 89.2% in the control treatment and 92.9% in the 1001 ngDvSnf7/g diet treatment. The overall mean number of viable eggs per female per day was calculated to be 18.2 in the control, and 20.3 in the DvSnf7 treatment. In addition, the mean reproductive performance (mean number of viable eggs/female/day) for the individual replicates from each treatment were compared statistically, and the test-item treatment did not differ significantly from the control (ANOVA followed by Dunnett's t-test, $\alpha = 0.05$). The mean reproductive performance was significantly reduced in the toxic reference treatment by 94.5% compared to the control. The study authors reported a NOEC of 1001 ng DvSnf7/g diet. In summary, when newly-emerged adults were fed for up to 18 days on a diet medium containing the nominal equivalent of 1001 ng DvSnf7 RNA/g diet (a mean of 771 ng DvSnf7/g diet as measured at Day 0), there were no adverse effects on survival or reproductive performance. This study was classified as acceptable.

Rove Beetle

A study was performed with the rove beetle to evaluate the potential effect of chronic dietary exposure to DvSnf7 on survival and reproductive success (i.e. number of F1 progeny). Testing was performed according to the guideline described by Grimm et al. (2000). DvSnf7 was incorporated into an artificial diet at a nominal concentration of 1000 ng DvSnf7/g diet. Diet was provided daily to adult *A. bilineata* (10 males and 10 females per replicate, 4 replicates per treatment) for 28 days, while the beetles were confined in boxes of damp sand. The survival of the beetles was assessed on three occasions over the four weeks (on Days 1, 7, and 28). Host insects (onion fly, *Delia antiqua*) were provided on Days 7, 14, and 21. The original adult beetles were removed on Day 28 and the numbers of new adults (the F1 progeny) that subsequently developed from parasitized fly pupae was recorded three times weekly until Day 70. A toxic reference treatment of teflubenzuron (an insect growth regulator) and a water-treated control were included in the bioassay.

On Day 28, DvSnf7 treatment and toxic reference mortality of the original adults was 11.3% and 5.0%, respectively. Neither of these differed significantly (based on Fisher's Exact Test, α = 0.05, from the control treatment mortality of 7.5%. The mean number of progeny produced per replicate was 991.8 in the control treatment, compared with 1028.0 in the 1000 ng DvSnf7/g diet treatment and 39.0 in the toxic reference treatment. Based on the nominal 1500 fly pupae provided per replicate for parasitization, these results equate to parasitism success of 66.1% in the control, 68.5% in the 1000 ng DvSnf7/g diet treatment and 2.6% in the toxic reference treatment. The study authors reported a NOEC of 1000 ng DvSnf7/g diet. Based on the results, there were no apparent treatment effects on the survival or reproductive success of the beetles when exposed to DvSnf7 at 1000 ng /g diet. This study was classified as acceptable.

Honey Bees

Two studies with honey bees were submitted to support the registration of MON 87411, including a study with adults (MRID 49315113) and another with larvae (MRID 49315112).

Honey Bee Adults

The study with adults evaluated the potential dietary effects of DvSnf7 on survival in a 14-day continuous feeding study. Newly emerged adults (≤ 2 day old) were exposed to DvSnf7 at a nominal concentration of 1000 ng DvSnf7/g diet., an assay control (no treatment), and a positive control (potassium arsenate at 20 µg/ml diet), each of which was present in a 50% sucrose/purified water (w/v) solution. Each treatment group included 80 adult bees in four replicates of 20 bees per replicate (one replicate per cage). Each cage was provided with approximately 10 ml of the appropriate treatment diet solution and bees were allowed to feed *ad libitum*. Bees from each treatment group were observed daily for mortality, abnormal behavior, and appearance. No adverse effects on survival, behavior and appearance were observed, and a NOEC for this study was reported to be 1000 ng of DvSnf7 test material/g diet. This study was originally classified as supplemental, as it was unclear whether food consumption was similar among all treatments. Clarification on this point was provided, and the study was upgraded to acceptable.

Honey Bee Larvae

In a 17-day dietary toxicity study, honey bee larvae were exposed to a single dose of DvSnf7 test material prepared at a nominal concentration of 1000 ng/g and delivered in a 10 µl aliquot of 30% (w/v) sucrose/purified water to each larval cell for a total mass of 11.3 ng DvSnf7/cell. Additional treatment groups included an assay control (30% sucrose solution) and a positive control (30% solution containing potassium arsenate at 2,000 µg/ml). Larva (2 to 3 days old) in all groups were exposed to a single 10 µl dose of the appropriate treatment at study initiation and observed over the course of larval/pupal development and adult emergence. Each treatment group consisted of 80 honey bee larvae in four replicates of 20 honey bee larvae per replicate. There was 100% survival in honey bee larvae treated with either DvSnf7 or the assay control. Emergence of adult honey bees from the test larvae in both the DvSnf7 and the assay control was initiated on the same day (Day 14), reached approximately 50% on Day 15, and reached 100% on Day 17 in both treatments. There was no survival in the positive control confirming the

validity of the exposure system. Observations of newly emerged adult bees indicated no adverse behavioral or morphological effects. Thus, the NOEL for this study was reported to be 11.3 ng of DvSnf7 per larva (fed a concentration of 1000 ng DvSnf7/g diet). This study is consistent with similar studies previously accepted for PIPs by EPA, and was thus classified as acceptable.

Soil Invertebrates

Earthworm

In a 14-day contact study, ten adult earthworms (*E. andrei*) were exposed to a single application of DvSnf7 incorporated into an artificial soil substrate at a rate equivalent to 5000 ng DvSnf7 per g soil dry weight. No food was provided during the duration of the test. The test treatment was compared to a control treatment of purified water added to the soil. Mortality was assessed over the 14-day testing period, and the change in fresh weight of the worms was assessed for survivors at 14 days after treatment. There was no observed mortality after 14 days. The mean percentage change in biomass was -9.4% and -8.4% in the control groups and treatment group, respectively. The study authors reported a NOEC of 5000 ng DvSnf7/g soil. This study was initially classified as supplemental but upgradeable. This guideline (OECD Guideline 207) is intended to test toxicity to earthworms via contact exposure, and it is unclear whether this route of exposure is appropriate for toxicity testing with double stranded RNA. Also, it is unclear whether the DvSnf7 test material would have degraded during the 14 day exposure period, and there is no assurance that the concentration in the soil was consistent throughout the study. The guideline also states specifically that multiple concentrations must be tested to establish a NOEC. Additional information was provided; however, it was determined that the study is not necessarily a good test of toxicity to this organism, since the DvSnf7 likely broke down very quickly in the test matrix, and because it is uncertain whether contact is an appropriate route of exposure. The study does show that short-term contact exposure at a relatively high soil concentration did not result in adverse effects as measured. However, the study is classified as supplemental, and is likely of limited utility to this risk assessment.

Springtail

Toxicity to soil invertebrates was also tested with a 28-day dietary toxicity study of DvSnf7 to springtails (*Folsomia candida*). DvSnf7 test material was incorporated into an inactivated yeast medium diet with a concentration of 1000 ng DvSnf7 per gram of diet. This test diet was compared to an untreated control diet of inactivated yeast, a blank (no food) diet, and a toxic reference diet (inactivated yeast treated with the insect growth regulator, teflubenzuron). Springtails were allowed to feed *ad libitum*. At 28 days, there were no significant effects on the survival or reproduction of the DvSnf7 treatment group compared to controls. Mortality in the untreated control, blank diet, and reference diet was 0%, 0%, and 93%, respectively. The mean number of progeny in the treatment groups was 169 for the untreated control diet, 0 for the blank diet, 167 for the DvSnf7 diet, and 0.3 for the reference diet. The NOEC, based on mortality, was reported as 1000 ng DvSnf7 per gram of diet according to the study report. This study was originally submitted to support the seed increase registration for MON 87411, and was initially determined to be supplemental, but upgradeable, since data to confirm food consumption was not

included, nor were raw data on mortality and reproduction. Clarifying information and raw data were provided and the study was upgraded to acceptable.

Field Evaluation of Arthropod Abundance

A field study (MRID 49553304) was conducted to determine whether MON 87411 maize produces adverse effects on nontarget insect populations across three geographic locations: United States, Argentina, and Brazil. Four to six sites were chosen within each geographic location, and within each site MON 87411 and a conventional control (with similar genetic background except for the insecticidal and herbicide tolerant traits) were tested for differences in nontarget arthropod abundance and nontarget pest damage. Each site included plots with MON 87411, the conventional control, and four conventional reference maize varieties (which varied across sites). At each site, nontarget arthropod abundance was determined using both sticky traps and visual counts, and counts were conducted multiple times over the growing season. Pest damage was also assessed using methods specific to each pest, and damage was assessed several times over the growing season. Out of 121 individual site comparisons, only five significant differences were detected between MON 87411 and the conventional control in nontarget arthropod abundance. For pest damage, 56 comparisons were made, and significant differences between MON 87411 and the conventional control were detected in three. These differences were determined not to be biologically significant.

This study was originally submitted to support the seed increase registration for MON 87411. EPA determined that the study provides some information on potential effects to arthropods in a more realistic field setting, but that the study design presents uncertainties in the data, which limits conclusions that can be drawn. It was unclear that the study, as designed, would detect differences and provide strong indication that adverse effects do not occur in the field. Some additional explanation was provided to address certain limitations; however, at issue are primarily the small plot sizes and unknown distances between them. Plot sizes were small, and it is unclear whether the plots were immediately adjacent or separated within the site. Monsanto Company referenced Prasifka et al. (2005), which tested the effect of multiple plot sizes (minimum size of 9 m x 9 m), and multiple separation distances (minimum distance of 45 m), on detection of effects to arthropods in field studies. At one site in Monsanto Company's study, plots had sides measuring as small as 5 m, which could easily be crossed by insects of relatively low mobility. The Prasifka et al. (2005) paper emphasized the importance of plot size when effects are not expected, as well as the need to include sufficient distance to ensure independence. Monsanto Company clarified the plot sizes used, but did not clarify the distance between plots this is important for understanding the independence of the data between treatments, since more mobile arthropods could move between plots with different treatments. It is also important in understanding the potential impact of insects from outside the test area moving into it, as this might dilute any effects that might be observed.

Initially, it was also unclear what other pesticide treatments were applied that could have affected the detection of differences in pest damage, and Monsanto Company clarified that all plots were treated equally, and that pest damage assessments were performed later in the season when pesticide use was minimal in the experimental area. Pest damage information is helpful as an additional line of evidence to show that the DvSnf7 did not affect pests known to be

insensitive to it; however, pest damage is not necessarily a definitive measure of toxicity since the fate of the pest insects is not known.

The data from this study provide some indication that adverse effects to nontarget arthropods in the field may not be highly likely. However, the results are relatively weak compared to a hypothesis driven field study conducted at an appropriate scale, and a field study with a more robust design would be needed to demonstrate unequivocally a lack of effects in the field. This study was determined to be supplemental.

7. Nontarget Organism Bioinformatic Analysis

At the request of EPA, Monsanto Company submitted a bioinformatic evaluation of the DvSnf7 sequence, wherein the 240 nt DvSnf7 sequence was compared to DNA transcriptome databases for 23 nontarget organisms (MRID 49553306). The organisms were chosen based on the criteria of plausible exposure to MON 87411 corn, availability of public genomes, and potential susceptibility based on current knowledge from laboratory bioassays. The evaluation was conducted using STELLAR software version 1.3 (Kehr et al. 2011) to identify exact 21 nucleotide matches between the DvSnf7 query and sequences contained in collections for the nontarget organisms, which were: *Anas platyrhynchos* (mallard), *Apis mellifera* (honey bee), *Bombus terrestris* (bumble bee), *Bos taurus* (cattle), *Caenorhabditis elegans* (nematode), *Canis familiaris* (domestic dog), *Columba livia* (rock pigeon), *Danaus plexippus* (monarch butterfly), *Danio rerio* (zebra fish), *Daphnia pulex* (water flea), *Drosophila melanogaster* (common fruit fly), *Equus caballus* (horse), *Gallus gallus* (red junglefowl/chicken), *Hyalella azteca* (scud), *Locust migratoria* (migratory locust), *Megachile rotundata* (alfalfa leafcutter bee), *Mus musculus* (house mouse), *Nasonia vitripennis* (jewel wasp), *Oryzias latipes* (medaka), *Rattus norvegicus* (Norway rat), *Solenopsis invicta* (red fire ant), *Sus scrofa* (pig), and *Tetranychus urticae* (spider mite). No exact matches of 21 nt or greater were found with any of the sequences searched.

This bioinformatic analysis provides some additional information toward another line of evidence of no expected adverse effects in nontarget organisms. Of these, the honey bee and chicken have also been tested against exposure to DvSnf7, and no adverse effects were identified in those studies. The report for the bioinformatic evaluation does not include searches of other coleopterans that would be more closely related to the target species. According to Monsanto Company (MRID 49745801), public databases only include the genome for the red flour beetle (*Tribolium castaneum*), and the analysis was not included because it was previously tested in a diet bioassay with DvSnf7 and no adverse effects were observed. Given that the same is true for the chicken and honey bee, and having both sets of information presents multiple lines of evidence toward determining the potential for effects, a bioinformatic analysis for DvSnf7 should include *T. castaneum*. While EPA is able to draw risk conclusions without this analysis, it would provide additional information that may serve to reduce uncertainty.

Bioinformatic analyses are not considered to be predictive of effects. EPA is currently evaluating how bioinformatics may be used in nontarget risk assessments, and at this time this analysis is considered to be supplemental information.

F. Nontarget Organism Risk Characterization

1. Birds and Mammals

EPA typically does not quantify exposure of nontarget vertebrates to PIP pesticidal substances, and instead relies on toxicity testing that is conducted at exposure levels reasonably expected to equal or, preferably, exceed maximum exposure levels in the field based on expression studies. Corn grain is the plant tissue that birds and mammals are most likely to consume, so if exposure assumptions are limited to grain only, then exposure is relatively simple to understand. However, it is likely that all species of birds and mammals that inhabit corn agroecosystems are exposed via other sources. While incidental dietary exposures to other corn plant material may occur, exposure through consumption of pest insects or other invertebrates that are insensitive to the toxins produced by MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn is another route by which birds and mammals may be exposed to DvSnf7. While DvSnf7 is not expected necessarily to accumulate within invertebrates, other plant materials that could potentially be consumed by these invertebrates show expression of DvSnf7 at levels several orders of magnitude higher than in grain. The level of exposure may not be significant; however, the extent of exposure via this source, or any other possible source, has not been determined. A "worst case" scenario that would reduce uncertainty would be based on the highest concentration shown to be expressed in the PIP plant based on expression studies submitted to EPA. To reduce uncertainty related to unknown levels of exposure, Tier I nontarget organism testing would then ideally be derived from the maximum expression level observed in any plant tissue, and not just grain.

Toxicity data available to assess risk for birds includes a 6-week study of broiler chickens fed a diet containing 57% MON 87411 grain. Since it is uncertain whether this level of exposure would represent some maximum, there is some uncertainty about whether this study would capture potential adverse effects. However, this study does provide longer term observations (compared to typical dietary studies) of sublethal effects on body growth at lower levels of exposure. Whether and how these measurements would relate to survival and reproduction in birds is unclear; however, they may be useful as indicators of subtle effects on growth or feed intake that might arise from exposure to DvSnf7, such as off-target effects.

The 14-day dietary toxicity study with Northern bobwhite exposed at 1000 µg DvSnf7/kg diet provides insight into potential effects at higher exposures. The LC_{50} determined from this study was > 1000 µg DvSnf7/kg diet. Based on the highest expression level measured in MON 87411 or MON 89034 x TC1507 x MON 87411 x DAS-59122-7 (0.097 µg DvSnf7/g dw leaf tissue), the level tested is 10.3 times the "worst case" exposure level expected. No lethal or sublethal effects were observed in this study. While the recommended limit concentration was not used, this study involved consumption of diet at 10 times the highest expected concentration in the field, which also occurred over 14 days. This concentration is based on dry weight measurements; the actual concentration would be lower in plant tissues or organs with their normal water content. Therefore, exposure was high relative to expected environmental concentrations, and represented potential effects of continuous exposure over many days. Since dsRNA effects on target species are known to be delayed by several days, it is not possible to know if additional effects would be observed beyond 14 days). This exposure is reasonable to

determine acute effects of DvSnf7 as expressed in MON 87411 and MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn. There are limitations in the applicability of this study to exposures to DvSnf7 at higher levels; however, for the current proposed registration, it does provide information to estimate potential effects to birds.

EPA does not expect adverse effects to occur to birds as a result of the proposed registration of MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn. The two studies discussed above provide a strong line of evidence that high exposures for a moderate duration and lower exposure for longer duration do not result in observable adverse effects on young birds. Bioinformatic analyses with red junglefowl/chicken, rock pigeon, and mallard indicated no exact 21 nt matches with the DvSnf7 sequence, providing an additional line of evidence toward expectation of no effects (EPA is cautious, however, in interpreting this information as predictive of effects).

Data available for mammals includes a 28-day study with mice, in which mice were dosed daily at levels up to 105 mg/kg/day, and a 90-day study with rats, which were fed MON 87411 corn grain at 33% of the diet for the duration of the study. Both studies indicated no adverse effects. The dose of 105 mg/kg/day likely exceeds maximum short term exposure levels to mammals that would occur in the field, and 90-day exposure to MON 87411 grain provide some insight into longer term exposures at low levels, similar to the 42-day study with birds. As with birds, bioinformatic analyses provides an additional line of evidence that the target gene does not exist in several mammalian species. This analysis involved several mammalian species, including cattle, domestic dog, horse, house mouse, Norway rat, and pig, and no exact 21 nt matches were found with the DvSnf7 sequence.

In the human heath review for DvSnf7 for the seed increase registration and for this proposed registration (USEPA 2015a, USEPA 2016), EPA discussed physiological barriers that exist that minimize exposure of humans. Such barriers include nucleases in saliva and the digestive tract (Park et al. 2006, Stevens and Hume 1995), acidic gut environments (Akhtar 2009, Loretz et al. 2006, O'Neill et al. 2011), membrane barriers, and rapid elimination from the blood (see USEPA 2016 and references therein). In fact, in the case of dsRNAs for therapeutic use, problems with delivery related to these barriers have been considered major obstacles in their development (Gavrilov and Saltzman 2012, Krieg 2011, Meade and Dowdy 2009). Birds, wild mammals, and other vertebrates would be expected to have similar barriers, and although there is likely some variation such that not all of them may be present, several of them would be. Therefore, even if high levels of DvSnf7 are consumed by birds and mammals, these barriers are expected to significantly limit uptake and the potential for effects.

Based on all of these lines of evidence, including the lack of effects observed in the available toxicity studies, bioinformatic analyses, and expected biological barriers to uptake, EPA does not anticipate that the DvSnf7 as expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn will result in adverse effects to birds and mammals.

One uncertainty with this conclusion would include potential sublethal effects that have not been measured in testing, such as reproduction. EPA had previously required an avian reproduction study to support the MON 87411 seed increase registration (requested in the deficiency letter), which would provide testing on additional sublethal effects. However, for DvSnf7 as expressed

in MON 89034 x TC1507 x MON 87411 x DAS-59122-7, EPA has determined that exposure to birds in the field is likely to be low, and would be counteracted by the barriers described above. This testing may be required in the future if information is received to conclude that exposure would be higher or that certain conditions may cause breakdown of one or more physiological barriers.

2. Freshwater Fish and Invertebrates

Exposure in aquatic environments is likely to occur in bodies of freshwater near corn growing areas, and may result from pollen drift and movement of leaf or other post-harvest crop residue off of cultivated fields. EPA has determined that due to very low levels of expression in pollen, contribution of pollen drift to aquatic exposure is expected to be minimal. Of greater concern would be post-harvest crop residues that enter water; however, as discussed above, senescent corn tissues or organs contain much lower amounts of DvSnf7 compared to the those same tissues or organs during the growing season, and leaching of the DvSnf7 is anticipated to occur, similar to what has been observed for Cry proteins. Once in the water, preliminary data have shown that it will not persist. Based on a standard pond scenario, "worst-case" estimates of DvSnf7 concentration in water range from 0.0014 – 0.0087 ng DvSnf7/mL using expression levels for stover and forage from Table 2. These concentrations are far below the dietary LC_{50}s for target insects, which are presumed to be most sensitive, since they have the gene targeted by DvSnf7. LC_{50}s for DvSnf7 in WCR and SCR were 1.2 ng/g diet and 4.4 ng/g diet, respectively (Bachman et al. 2013). NOEC values for less sensitive insects, some of which were closely related, ranged from 500 ng/g diet to 5000 ng/g diet. While toxicity values for insects are not normally compared to aquatic exposure estimates, these data show that DvSnf7 is highly specific for its targeted gene. Therefore, sensitivity in other organisms is expected to be lower. Since exposure values in water are two to three orders of magnitude below the LC_{50} for highly sensitive organisms, it can be concluded that adverse effects in less sensitive organisms are unlikely. More is discussed below about specificity, including potential for off-target effects.

The channel catfish study provides some information on the potential dietary toxicity of DvSnf7 to freshwater fish. Considering DvSnf7 expression in grain (0.104 ng DvSnf7/g dw grain), a diet consisting of 30% MON 87411 grain would have a concentration of DvSnf7 of 0.031 ng DvSnf7/g diet. This level is above estimated concentrations in water, but below concentrations in other corn tissues expected post-harvest. Therefore, this study may not have tested a maximum hazard level; however, it does show continuous exposure to low levels for 8 weeks did not affect survival, food consumption, weight gain, or diet conversion. Some of these sublethal effects may be sensitive to off-target or other unexpected effects potentially resulting from exposure to dsRNA. It is also noted that the bioinformatic analysis indicated no 21 nt sequence matches with DvSnf7 for two fish (zebra fish and medaka) and two aquatic invertebrates (water flea and scud). Additionally, some of the physiological barriers described above are likely present in freshwater fish, which would limit uptake and potential effects if exposure were to occur.

Based on the available information, adverse effects to freshwater fish and invertebrates are not expected to occur as a result of exposure to DvSnf7 as expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn. As with birds and mammals, conclusions are drawn from

expected low environmental exposure, specificity of the DvSnf7 for its target gene in the target pests, and the role of physiological barriers. Some uncertainty is associated with exposure assumptions (e.g., it is assumed to parallel what occurs for Cry proteins), specificity (e.g., off-target effects are unknown), and the existence of physiological barriers in fish. If information becomes available that changes these assumptions, additional data may be required, such as the fish life-cycle study noted above.

3. Marine and Estuarine Fish and Invertebrates

As described above, significant exposure to DvSnf7 is not expected in aquatic environments, including marine and estuarine systems. Therefore, adverse effects are not anticipated for fish or invertebrates in these environments.

4. Nontarget plants

Uptake and transport of dsRNA by plants has been documented (Hunter et al. 2012, Li et al. 2015); however, DvSnf7 is not expected to be present at high levels or to persist in soil such that significant uptake would occur. It is noted that in these studies where uptake was successful, application rates were relatively high compared to estimated soil concentrations for DvSnf7. For example, Li and colleagues (2015) describe soaking *Arabidopsis thaliana* roots in a solution of 1 mg dsRNA/mL or irrigating plants with 10 mL of a 0.5 mg dsRNA/mL solution to achieve significant gene knockdown. Hunter et al. (2012) describe a solution of 0.13 mg dsRNA/mL dsRNA (2 g in 15 L) added to soil as a root drench. Details of these studies are insufficient to estimate resulting soil concentrations (and are also not clear whether stabilizing or transfection agents may have been used); however, given that the worst case soil concentration estimate was calculated to be 0.571 ng DvSnf7/g, these studies likely represent exposure that is several orders of magnitude higher. Since exposure is expected to he low in soil environments, and because DvSnf7 is not likely to persist, significant uptake by plants is not anticipated. EPA recognizes that more study is needed on the potential uptake of dsRNA by plants.

5. Nontarget Insects and Other Invertebrates

Primary work to demonstrate specificity for the target pest is described in Bachman et al. (2013). In this study, bioassays were conducted with either DvSnf7 dsRNA or dsRNA developed from an ortholog of the *Snf7* gene in several close relatives. This study showed that DvSnf7 was only active within the Chrysomelidae Family of coleopterans, which provides a strong line of evidence toward the specificity of the intended effect of DvSnf7 on its target species.

Exposure of nontarget insects to PIP pesticidal substances is typically determined based on expression levels within plant tissues. Expression levels can differ between plant tissues and also over time, and the plant tissue with the most relevance to the insect diet is typically used to determine dosing levels or diet concentration in nontarget testing. Expression levels in pollen are often used, since many nontarget insects will consume pollen, and expression in leaf may also be considered. As described above for birds and mammals, there is some uncertainty with the actual exposure in the field given the varying diets within arthropod communities. Therefore, a conservative approach to estimating exposure of nontarget organisms to PIP pesticidal

substances would be to utilize the highest expression levels measured among all tissues based on dry weight measurements. While this may overestimate exposure for certain organisms, it reduces uncertainty around actual exposure in the field.

Nontarget insect studies submitted in support of this registration reported NOEC values of ≥1000 ng DvSnf7/g diet. In the environmental risk assessment submitted by Monsanto Company (MRID 49505802), NOEC values determined from nontarget testing were compared to maximum expected environmental concentrations (MEECs) to calculate margins of exposure (MOEs). If MOEs are >10 times the MEEC, then risk is assumed to be negligible. Utilizing the highest mean dry weight expression level measured (0.097 µg DvSnf7/g leaf tissue), NOEC values of 1000 ng DvSnf7/g diet determined in nontarget insect testing would produce a MOE of 10.3, which, if using this approach, would indicate a low likelihood of adverse effects. The MOE for bees would be > 3,400, assuming the maximum recorded expression in pollen represents the MEEC for honey bees (maximum dw value measured was 0.292×10^{-3} µg/g). For soil dwelling organisms, this level represents a MOE of > 1000 to > 3700, depending on the worst case estimate of soil concentration used. This approach has been found to be acceptable by EPA in previous risk assessments; however, given that the studies are maximum hazard concentration tests in which only one concentration was tested, there is uncertainty around the biological meaning of the NOEC. Without testing at additional higher concentrations, it is not possible to know how close the NOEC is to levels at which effects might be observed. Nonetheless, EPA considers level at which toxicity was tested to be sufficiently above expected environmental concentration, and that the studies submitted are reliable tests of potential effects to nontarget insects at realistic field exposure levels.

For the seed increase registration of MON 87411, only one study on nontarget arthropods involved measurements on reproduction. For the full commercial registration, EPA required at least two additional studies examining reproductive endpoints in nontarget insects. Therefore, in support of this proposed registration, there are studies on eight species of nontarget arthropods (including three coleopteran species, and two studies with honey bees), involving observations on mortality and sublethal effects including measures on biomass, development (emergence, time to emergence), and reproduction (egg production, egg viability, number of progeny). The duration of these studies ranged from 10 to 35 days, involving continuous exposure throughout the study in most cases. No adverse effects were observed on these endpoints in any of these studies. Combined, these studies provide strong evidence that adverse effects are unlikely in nontarget arthropods exposed at least 10 times the highest anticipated environmental exposure level.

Due to their potential to provide a food resource for vertebrates, a study with earthworms was also recommended by the 2014 SAP. A study with earthworms was submitted, and no adverse effects were observed as measured in the study; however, the study was conducted in such a way that exposed the test animals through contact exposure only. Additionally, even though the study was conducted over 14 days, it is likely that the DvSnf7 was not present for the duration of the study due to its breakdown in the test matrix. Therefore, the study is only useful to show the potential for adverse effects resulting from short term contact exposure. It is unclear whether this route of exposure is relevant for naked dsRNA in soil. More relevant testing with earthworms would involve oral exposure; however, such testing (OECD Guideline 222) is

conducted in a matrix that includes manure, which would have high levels of bacteria that would quickly break down non-stabilized dsRNA. Therefore, appropriate testing methods for earthworms may require more consideration for any future proposed dsRNA PIPs. As discussed above, DvSnf7 exposure levels expected in soil are low and degrade quickly, and may not impact invertebrates that would consume soil directly.

While not specifically submitted to address nontarget organism risk, EPA also notes that synergism data (described below in Section III) included tests with Colorado potato beetle (*Leptinotarsa decemlineata*, CPB) and European corn borer (*Ostrinia nubilalis*, ECB). These studies that examined mortality (CPB) and growth inhibition (ECB) with exposure to DvSnf7 in combination with Cry proteins expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7. Neither of these indicated adverse effects that differed from expected values. In ECB, no additional effects resulting from exposure to the coleopteran active PIP pesticidal substances tested, including DvSnf7.

Some studies of dsRNA in invertebrates show the existence of physiological barriers that can significantly limit uptake in insects. These barriers include ribonucleases in saliva (Allen and Walker 2012, Christiaens et al. 2014) and DNA/RNA non-specific nucleases in the hemolymph (Garbutt et al. 2013). Other evidence of some barriers are demonstrated in studies requiring certain conditions for delivery of dsRNA, such as by microinjection (Whyard et al. 2009) or nanoparticle encapsulation (Sarathi et al. 2008). While these barriers are not fully understood, and information is not yet available to extrapolate between insect taxa, it is apparent that such barriers reduce bioavailability in some insects. However, at this point, knowledge about these barriers does not permit generalization across insect or other arthropod taxa, so use of this information in drawing conclusions about risk is limited.

Based on the specificity of the intended effect and the lack of adverse effects observed through testing with several nontarget arthropod species, EPA concludes that adverse effects to nontarget insects and other terrestrial invertebrates are not expected to occur as a result of exposure to DvSnf7 as expressed in MON 89034 x TC1507 X MON 87411 x DAS-59122-7 corn. Honey bees and other pollinators are included in this conclusion, and the results of testing with both larvae and adult honey bees provide strong evidence in support of a conclusion that adverse effects are not anticipated. Additionally, Monsanto Company has provided results of bioinformatic analyses with both honey bee and humble bee, neither of which identified exact 21 nt matches with DvSnf7. As noted above, these data provide supplemental information to support this conclusion, but EPA recognizes that they are not necessarily predictive of effects.

One uncertainty that remains after testing with DvSnf7 is the potential for systemic RNAi that could lead to effects on offspring. For example, Terenius et al. (2011) describe work with *Hyalophora cecropia* in which RNAi was observed in the offspring of insects injected as pupae, indicating uptake of the dsRNA by oocytes in the pupae. Khajura et al. (2015) also observed reduced hatching, but not reduced egg laying, in WCR adults that were fed dsRNAs targeting two developmental genes. It was unclear whether the effect, which was the intended effect and not a result of off-target silencing, resulted from gene knockdown in the tissues of the parents or the offspring, or whether siRNAs were formed in the cells of initial uptake and then translocated. On the other hand, Whyard et al. (2009) failed to observe systemic RNAi in *Drosophila*

melanogaster; therefore, systemic RNAi may be possible in some species but not others. The mechanisms driving the systemic RNAi among species are not known, nor is the potential for it to cause realistic concerns when silencing of target genes are not related to development and other off-target effects are not apparent. Therefore, for DvSnf7 potential effects on the progeny of nontarget organisms are not expected; however, these effects should remain a potential consideration for ecological risk assessment.

G. Off-Target and Other Unintended Effects of DvSnf7

As discussed in EPA's white paper presented to the SAP in 2014 (USEPA 2013), exposure to dsRNA may bring about unintended effects, such as immune stimulation, over-saturation of RNAi machinery, and off-target effects. Of these, off-target effects are more likely to be of greater concern, particularly for nontarget invertebrates with potential for greater exposure. As discussed above, off-target effects may result from binding of siRNAs to genes other than the target gene, and appear to be related to partial sequence homology between the "seed region" (positions 2-7 or 2-8) of the siRNA and the 3' untranslated region of messenger RNA transcribed from a nontarget gene (Birmingham et al. 2006, Jackson et al. 2003), though the process is not fully understood (Birmingham et al. 2006, Kamola et al. 2015). Off-target effects are siRNA specific, instead of target-gene specific, and may influence other signaling and transcription pathways (Jackson et al. 2003). They have also been shown to reduce cell viability in controlled studies (Fedorov et al. 2006). Off-target effects may not be severe or prolonged if they were to occur, but their effects would depend on the sensitivity of the nontarget organism, the gene(s) targeted, and the potential for amplification of siRNAs (e.g., Sijen at al. 2001, Tomoyasu et al. 2008). While nontarget organisms do not have the gene specifically targeted by the DvSnf7 dsRNA, off-target effects present an uncertainty for nontarget organism risk, since they may potentially result is unpredicted downregulation of genes within the nontarget organisms' genomes.

Off-target effects are considered artifacts of *in vitro* testing in studies of functional genomics that lead to false positives and potential erroneous results. However, off-target effects are also recognized as potential liabilities in the therapeutic use of siRNA (Gavrilov and Saltzman 2012, Kamola et al. 2015, Krieg 2011), which may also indicate concern for nontarget organisms. For nontarget species, off-target effects have been primarily observed in *in vitro* studies involving tissues of nontarget species (Jarosch and Moritz 2012; Oates et al. 2000, Zhao et al. 2001). One study noted effects observed *in vivo*; however, the study is not well described, and it involved the use of dsRNA sequences with potentially up to 20 bp sequence homology with the test organism genome, most of which were derived from the tested organism (Jarosch and Moritz 2012). Therefore, little information is available about the realistic impacts of off-target effects on nontarget organisms.

Without further in vitro testing to observe more directly the potential for off-target silencing, EPA cannot discount the possibility that these effects occur with exposure to DvSnf7. Off-target and other unintended effects from dsRNA may result in a range of biological consequences, and may be more likely to be observed in nontarget organisms that are more closely related to the target pest. Therefore, EPA required additional testing on reproductive effects of DvSnf7 in nontarget insects to provide data on additional endpoints for more closely related organisms.

Previously only one study included these endpoints, which are critical to understanding impacts to individuals, as well as impacts at the population level and higher. With these additional studies, the nontarget data available for DvSnf7 includes testing across a wide range of taxa, with additional and more intensive focus on species most likely to be impacted (insects and other arthropods). Testing has also been performed at high concentrations, with continuous dietary exposure over many days and with study durations that are considered reasonably sufficient to allow observation of adverse effects. No apparent adverse effects have been observed. Therefore, EPA concludes that unintended effects, if they occur, are unlikely to be of biological significance for DvSnf7 as expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn.

This conclusion does not necessarily mean that such effects do not occur. It is possible that they do not occur with exposure to DvSnf7; however, it is also possible that they do occur but do not cause phenotypic changes that result in adverse biological consequences. If the latter, this lack of observed effects may be due to the specific genes that are downregulated as a result of off-target binding, or, since off-target effects are concentration dependent, may be owed simply to the levels of exposure tested. Therefore, these conclusions for DvSnf7 as expressed in MON 87411 and MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn would not necessarily be applicable to other event combinations with MON 87411 that result in higher expression levels of DvSnf7.

H. Risk Assessment Conclusions for DvSnf7

Based on the data presented and anticipated minimal exposure in certain environments, adverse effects to nontarget organisms are not expected as a result of DvSnf7 as expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn. Data provided to EPA show that the intended effect of downregulation of the *Snf7* gene is likely only to be observed in the target insect and very close relatives, and unintended effects of dsRNA, like those discussed at the 2014 SAP, appear to be unlikely, or do not cause significant biological consequences, at exposure levels expected in the environment.

The 2014 SAP report outlined potential scenarios that could minimize concern for nontarget exposure and effects related to dsRNA PIPs, which included scenarios in which:

1. The dsRNA PIP is very specific without homology to any of the genomics entries in sequence databases
2. The dsRNA PIP is not modified and therefore likely to degrade rapidly in the environment
3. The dsRNA PIP is expressed at extremely low levels and is tissue specific in its site of production.

DvSnf7 has been shown to be very specific in its intended effect in the target insect and very close relatives, and a search a limited number of databases for nontarget organisms across a wide taxonomic range did not find exact matches of 21 nt or greater with any of the sequences searched. This search should be expanded as more databases for nontargets become available. DvSnf7 is also not modified in such a way to he more stable in the environment, and its hairpin

structure is such that would not provide added environmental stability (e.g., as may be seen with dsRNA with multiple stem-and-loop structures). Degradation data presented also show that it degrades rapidly in soil, and preliminary data indicate that it will not persist in water. While it is not tissue specific, DvSnf7 also is expressed at extremely low levels. It does function as a pesticide at these levels; however, its expression is much reduced over the season and likely degrades further *in planta* at senescence (see Blank and McKeon 1991, Miller et al. 1999). In most cases when it is released into the surrounding environment from plant tissues, its concentrations are expected to be very low. Therefore, based on these characteristics, and the data available that show minimal nontarget hazard, EPA concludes that DvSnf7 meets the majority of these criteria, and should be considered to have minimal risk to nontarget organisms and the environment.

I. Discussion of 2014 SAP Recommendations with Respect to DvSnf7

The 2014 SAP provided recommendations for assessing ecological risks of dsRNA based pesticides, many of which are applicable to this assessment for DvSnf7. The charge questions related to the environmental fate of dsRNAs, barriers to uptake, unintended effects, and data requirements and a framework for nontarget testing. The discussion that follows describes the advice provided by the SAP and how that advice has been used in this risk assessment. The discussion does not include advice that was specifically given for exogenously applied dsRNA based pesticides.

1. Environmental Fate of dsRNAs

SAP Recommendations

• EPA requested advice on the extent to which data on soil degradation would inform EPA on nontarget exposure to dsRNA PIPs. The SAP responded that at the time there was little information to evaluate degradation, persistence, and bioavailability of dsRNA and siRNA in the environment. Any testing would need to involve the dsRNA produced in the plant as well as any RNAi effectors (e.g., siRNAs that may silence genes), and should consider bioavailability and pesticidal activity. The panel recommended consideration for biotic and abiotic factors (e.g., cold temperatures, reduced microbial activity) to prolong availability in the environment.

• EPA also requested advice on additional testing that may be required to fully understand the environmental fate of dsRNAs. The SAP responded that in addition to soil degradation, degradation within plants and off-site movement of plant material should be known. Soil studies should take into consideration a range of conditions potentially encountered, and the potential for transfer of the dsRNA to nontarget organisms. The SAP urged caution in utilizing environmental models to predict exposure until more empirical fate data could be gathered. Representatives from Monsanto Company had provided information on soil degradation for DvSnf7, which the SAP noted had been published by the time the SAP report was finalized. The panel recommended a similar study for determining soil fate of dsRNAs.

EPA Response

With respect to the DvSnf7 PIP, the study presented by Monsanto Company was the soil degradation study submitted in support of this registration, and EPA determined this study to be acceptable. This study includes both an examination of degradation using molecular means of analyses, as well as a concurrent bioassay with a sensitive pest species to confirm the loss of bioavailability and/or bioactivity of the DvSnf7 and the rate at which this occurs. Both types of analyses indicated that DvSnf7 degrades rapidly and loses bioactivity, and would not be available for transfer to higher trophic levels. While no study was submitted to describe all possible degradation products of the DvSnf7 dsRNA, EPA has determined that such information is not needed, since the study submitted essentially tests the potential persistence of these products. Cold temperatures were not specifically examined, though EPA agrees that these conditions would likely prolong persistence. However, based on plant expression data, levels of DvSnf7 remaining after harvest are anticipated to be low.

Plant expression data are available that show expression levels over time, and these data indicate that over the course of the growing season, DvSnf7 expression levels decline and are several orders of magnitude lower at senescence compared to highest exposures in the early season. Additional data on plant tissue movement would not necessarily aid this screening level risk assessment, since generic assumptions lead to the conclusion that the final disposition of the DvSnf7 is in the soil in terrestrial environments, and in the water in aquatic environments no matter where the plant material may move. EPA also has access to preliminary information on the potential for persistence of DvSnf7 in water. EPA agrees that at this point there is not sufficient information to utilize any environmental fate models to examine the fate of DvSnf7 in soil or water, and believes that the current set of information indicates that DvSnf7 does not persist in the environment, and would not be present at high levels in these environmental matrices.

2. Barriers to Uptake

SAP Recommendations

• EPA requested information on factors that may play a role in uptake within the gut of animals and potentially limit exposure to dsRNA, and how these barriers may be generalized across taxa. The SAP responded to the first request with comments on a range of harriers tbat have been described in the literature, including the influence of tissue/cell specificity, transport proteins, dsRNA length, dose, dsRNA pesticide formulation, metabolic degradation, and the peritrophic matrix lining the midgut of many insects. The SAP commented that published literature indicates a wide range of environmental uptake mechanisms that vary between species and across taxa, and that at present there is insufficient understanding of the uptake mechanisms of dsRNAs to allow for generalization across species.

• EPA also requested information on the significance of the contact route of exposure for nontarget organisms, and what barriers would exist for this route specifically. The SAP responded that studies documenting successful induction of RNAi following exposure via contact involved soaking test organisms in media containing dsRNA. There is limited

information about the details of the exposure, but it appears in these cases that the dietary route was probably the primary route leading to the observed activity. The SAP concluded that the probable route in most cases is through the diet, but that contact exposure should be considered on a case-by-case basis. They also concluded that there is not enough information on potential barriers that limit uptake with contact exposure, and generalizations are not possible.

EPA Response

This response is limited to barriers that may exist to naked dsRNA, such as DvSnf7, and not dsRNA that is stabilized or enhanced for uptake. EPA agrees that variation is expected among barriers to uptake of dsRNA; however, certain common physiological characteristics may indicate common barriers across taxa of vertebrates. For example, birds and mammals both have acidic stomachs and RNases in their blood. Barriers to uptake related to the dsRNA (e.g., its large molecular size and hydrophilicity) will also have applicability across wide ranging organisms with similar tissue types. While these characteristics may vary, it is likely that redundant barriers exist within vertebrates to dsRNA like DvSnf7. Therefore, for vertebrate species, EPA is confident that these barriers contribute a line of evidence supporting a conclusion that adverse effects are not expected for vertebrates. Since these barriers are less well characterized for nontarget vertebrates other than humans, other information, including results of toxicity testing and bioinformatic analyses, are still needed to support risk conclusions. EPA agrees that such barriers are not well characterized for invertebrates, and there is less confidence in making generalizations between species and across other taxa regarding barriers to uptake.

For DvSnf7, the contact route of exposure is not expected to play a large role in exposure of nontarget organisms, especially since exposure levels expected in the environment are anticipated to be low and DvSnf7 is not expected to persist. EPA agrees that contact exposure should be considered on a case-by-case basis, and that DvSnf7 does not present a situation that warrants this consideration.

3. Unintended Effects of dsRNA

SAP Recommendations

• EPA asked the SAP to comment on the nature, including the biological significance, and likelihood of unintended effects that might be reasonably anticipated for nontarget organisms exposed to dsRNAs. The SAP limited its discussion on unintended effects to considerations for nontarget insects. This reasoning for this limitation was not explained in the report. The SAP commented that unintended effects due to agricultural uses of dsRNAs may be likely due to the potential scale of application, but noted that specificity could limit effects observed. The SAP noted cases in which saturation of RNAi machinery and immunostimulation have been observed in mice or cell cultures, but that these require a fairly high dose to cause deleterious effects and have not been noted in invertebrates to date. The dose required for insects is not known, but may have significant consequences. Off-target effects have been documented in many laboratory studies, and may be a concern for nontarget organisms; however, knowledge gaps prevent prediction of such effects with certainty.

• EPA also requested advice on additional information that could be required to characterize these effects and how these data would reduce uncertainty in risk conclusions. The SAP primarily referred to their response to EPA's question on data requirements to address this question. Their advice emphasized characterization of exposure in designing testing requirements. More is discussed on this advice below.

EPA Response

Because they are assumed to have the greatest potential for exposure to DvSnf7, EPA agrees that consideration for unintended effects should be focused primarily on nontarget insects. EPA believes that sufficient evidence has been submitted to conclude that the intended effect of DvSnf7 dsRNA in its target species is highly specific. Therefore, silencing of the target gene in nontarget organisms is not expected. As for other unintended effects described above, without extensive *in vitro* testing, EPA believes that it is not possible to discern whether any adverse effects are due to off-target effects, saturation of RNAi machinery, or immunostimulation. However, it is expected that studies of sufficient exposure and duration that also examine endpoints likely to be sensitive to biological consequences of these unintended effects, would indicate whether such biological consequences are likely to occur. Given the range of testing with nontarget insects, as described above, EPA has concluded that such effects related to DvSnf7 exposure, if they do occur, do not occur at levels that cause significant biological consequences. EPA agrees that generalizing the potential for these effects across taxa is not possible at this time for dsRNA.

4. Data Requirements and Framework for Nontarget Testing

SAP Recommendation (a)

EPA requested that the SAP comment on how the current PIP framework for nontarget testing (based primarily on experience with *Bt* derived proteins) will inform risk assessment for dsRNA-based PIPs, paying particular attention to the potential for unintended, latent, and/or chronic effects, and appropriate life stages for testing. The SAP provided a general response that emphasized characterization of exposure (soil degradation, in planta exposure, off-site movement) for determining potential risk of dsRNAs to nontarget organisms. The SAP also described limitations in the current framework that affect the ability to characterize the potential for effects of dsRNA on nontarget organisms (i.e., related to limited understanding of exposure, persistence, specificity, mechanisms of action, plant tissue expression, dose needed to trigger RNAi, RNAi amplification, and duration of the effect). The SAP noted that the most difficult effect to predict would be off-target effects, noting that timing and duration of studies may not capture all potential off-target effects.

EPA Response

EPA's risk assessment for DvSnf7 has focused extensively on exposure, including anticipated movement of PIP plant material and DvSnf7 in the environment, degradation in soil and water, and plant expression of DvSnf7 in various tissues and organs over the course of the corn growing season. Much of this information addresses some of the limitations related to characterization

described above. The dose needed to trigger RNAi has not been characterized with respect to the number of molecules. However, the dietary concentration required to elicit the intended response in target organisms has been characterized, and this effect has also been shown not to occur in related organisms at much higher exposure levels (Bachman et al. 2013). Since the intended effect is mortality, it has not been possible to determine the duration of this effect; however, it is noted that dsRNA has the effect of a gene knockdown, and not a gene knockout, so except in cases where siRNAs are amplified within the organisms, cessation of exposure is expected to result in reduction and eventual cessation of effects. This is also expected to be true for off-target effects.

EPA agrees that testing for off-target effects requires studies that evaluate multiple endpoints and life stages, and that these studies also must be of sufficient duration or timing to observe the effect. Not all testing performed for DvSnf7 has included the entire life cycle of nontarget organisms, which does present some uncertainty, but insect testing has been done on a range of species; at concentrations well above environmental exposure; has included several life stages; has been conducted over durations that are considered to be sufficient to observe significant gene knockdown; and have included lethal, sublethal, and chronic endpoints. Many genes may be repressed by off-target gene silencing resulting from exposure to dsRNA (Boettcher and McManus 2015, Gumienny and Zavolan 2015). Therefore, it seems likely that unexpected effects would have been observed in at least one of the studies reviewed for DvSnf7 if they do occur and cause adverse biological consequences.

SAP Recommendation (b)

EPA requested advice on additional nontarget effects testing and/or other testing approaches (e.g., bioinformatics) that should be considered, and how these would reduce uncertainty. The SAP responded with general advice, including:

- Bioassays should include doses that mimic *in planta* expression and use plant tissue where possible
- Chronic and sublethal effects should also be considered in addition to mortality
- Testing should include entire life cycles, and examine multiple life history parameters and should be conducted as much as possible under realistic conditions, which will need to be based on strategic research to determine what life stages should be assayed
- Testing should include an immunological endpoint and an endpoint to measure saturation of RNAi
- Testing should include possible synergistic effects and potential for evolution of pest resistance

EPA Response

Much of EPA's response to SAP recommendation (a) applies to this point as well. Several of these points have been incorporated into the ecological risk assessment of DvSnf7. EPA typically requires exposure levels in nontarget studies to be above *in planta* expression levels to account for uncertainties related to environmental exposure. However, plant material is accepted as a test material for nontarget testing, and has been used in studies to support the risk

assessment of DvSnf7. While no lifecycle testing has been done, testing has included several life stages, though it is noted also that the SAP report recommends testing entire life cycles as well as determining the most appropriate life stage to test. Additionally, tests have included chronic, sublethal, and lethal endpoints, and synergism testing has been done with DvSnf7 and other PIPs expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn.

Not all of the above (or below) studies are necessarily available, widely used, and/or validated. Lifecycle studies with insect species not typically used in laboratory settings would likely require protocol development and validation. Traditional nontarget testing could be modified to include examination of gene expression (e.g., see Vélez et al. 2016), though which genes to test would need to be determined as well as how to interpret the results of such studies when gene knockdown is observed with no accompanying overt adverse biological effects. Many of these suggestions are valid considerations for testing, but it is not clear that all of them are necessarily needed in order to assess nontarget risks (see below for further discussion), and some may require considerable time to develop.

EPA agrees that the development of pest resistance is a concern for DvSnf7, but this type of analysis is not included in PIP ecological risk assessments. These assessments are performed separately, and further discussion of this point is not included in this memorandum.

SAP Recommendation (c)

The SAP also responded by providing a description of an exposure-based conceptual framework for testing with the following steps:

1) Characterize the dsRNA: Characterization should include dsRNA sequence, target gene, expected phenotypic effect, how the effect is measured and its duration, target organism, dose to trigger an effect in the target organism, expression level for PIP pesticidal substances, and degradation kinetics in decaying plant tissue.

2) Identify species exposed: Identification of non-target species for testing would occur by examining results of step #1, plus probable routes of exposure and trophic linkages. Based on this curtailed community, *in silico* searches are then performed to refine requirements for specific *in vitro* studies (e.g., presence of dsRNA/siRNA in cells of exposed nontarget species).

3) Conduct model feeding studies: Feeding studies are then conducted on nontarget organisms based on *in silico* searches, and they would examine effects of factors described above such as RNAi saturation, the dose required to achieve an effect, and effects on different life stages and tissues. Protein production of off-target genes should be observed, and subsequent tests are performed to determine whether phenotypic changes are measurable. These changes include lethality and sublethal effects. This step also includes determination of the potential for synergism and resistance development.

4) Conduct cellular and molecular studies: These studies are then done to examine whether effects observed result from differential gene regulation, and if so, to identify the genes and the dose. The persistence of the effect and the mechanism (RNAi, oversaturation of RNAi machinery, immunostimulation) is then determined. Further analysis is done to examine how the dsRNA is taken up by cells.

5) Determine population level effects: This step includes conducting field studies over multiple years and examining food web disturbances.

6) Determine mitigations that limit uptake: This step involves instituting mitigations or re-designing the dsRNA to minimize off-target effects.

EPA Response

In general, many of the above analyses have been included in the DvSnf7 risk assessment. Some of these suggestions are more academic in nature. They would help to increase understanding of the potential effects of RNAi and the underlying mechanisms involved, but testing more typical of ecological risk assessments for PIPs, with expansion involving additional endpoints, would provide sufficient information to allow EPA to conduct a risk assessment. Specifically in response to the steps involved:

1) Characterize the dsRNA: Much of the characterization described for this step is part of the product characterization and environmental fate testing for PIPs. Specific to DvSnf7, these studies have been submitted and evaluated. The duration of the intended effect in the target pest was not included; however, since the intended effect (mortality) has only been observed in the target species and very close relatives, this information is not considered necessary for the review of DvSnf7 as expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn.

2) Identify species exposed: This step involves determining which species are likely to be exposed. While EPA has the ability to determine these species generically, determining the makeup of nontarget communities across all areas where corn may be grown, such that individual local species are identified for testing, is beyond the scope of screening level risk assessments and are not considered necessary for determining nontarget risk. *In vitro* studies would be helpful for understanding the underlying mechanisms of unintended effects; however, ultimately, toxicity testing provides the information needed for nontarget risk assessment, so if these studies sufficiently test the biological consequences of these effects, then these should be sufficient for nontarget risk assessment.

3) Conduct model feeding studies: This step involves feeding bioassays based on a refined set of species determined from testing in step #2. EPA agrees that incorporating additional endpoints would expand the understanding of potential effects of dsRNAs; however, certain studies, such as testing protein production from off-target genes, would require development to target the test for an appropriate set of genes. This step concludes with testing to examine a battery of lethal and sublethal

effects, which presumably would confirm effects and/or determine their biological significance. This testing has been done with DvSnf7. While the species tested cannot be refined further for DvSnf7 as suggested for step #2, testing was done with a range of species without observation of adverse effects. If significant effects are likely to occur with exposure to DvSnf7 at the exposure levels tested, then they would likely have been observed in these studies, and the recommended *in vitro* studies would not be needed to determine risk. Therefore, for DvSnf7 expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn, EPA considers results of this testing to be sufficient and conclusive.

4) Conduct cellular and molecular studies: While these would be helpful in understanding underlying mechanisms, if effects are not observed in testing (as with DvSnf7), then this additional testing would not be needed. All potential mechanisms for lethal, sublethal, and chronic effects are not necessarily known for all pesticides, and would not need to be if the effect could be predicted relative to environmental exposure, thereby allowing estimation of risk.

5) Determine population level effects: Confirmatory field studies have been required for *Bt* based PIPs in past registrations, but are no longer considered to be necessary for risk assessment. Similar data may reduce uncertainty with the conclusions of DvSnf7; however, such studies would need to be carefully designed and hypothesis driven, not just censuses of insects in fields with and without DvSnf7. At this time, EPA is not requiring additional field testing because the body of evidence available is sufficient to determine nontarget risks.

6) Determine mitigations that limit uptake: Nontarget exposure to DvSnf7 is limited, effects are not expected, and no risk has been identified. Therefore, mitigation of risk is not necessary given the current set of information.

III. Ecological Risk Assessment for Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, and Cry34/35Ab1 as Expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 Corn

A. Description and Regulatory Background of Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, and Cry34/35Ab1 in MON 89034, TC1507, MON 87411 and DAS-59122-7

The *Bacillus thuringiensis* derived Cry proteins in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 are expressed in other single- and combined-trait corn products, including SmartStax (MON 89034 x TC1507 x MON 88017 x DAS-59122-7; EPA Reg. No. 524-581), in which they are expressed together. EPA has previously determined that these proteins do not present risks to nontarget organisms and the environment. Data developed on the individual proteins expressed in these events is being bridged to support the registration of MON 89034 x TC1507 x MON 87411 x DAS-59122-7.

MON 89034 produces Cry1A.105 and Cry2Ab2, which are insecticidal proteins that protect against feeding damage caused by ECB and other lepidopteran insect pests. Cry2Ab2 is derived

from *Bt* subsp. *kurstaki*, whereas Cry1A.105 is a modified *Bt* Cry1A protein. The ecological risk assessment for event MON 89034 corn was most recently updated in 2010 (USEPA 2010c).

TC1507 produces the *Bt* subsp. *aizawai* Cry1F protein to control larvae of the European corn borer and other lepidopteran insect pests. A complete summary of the data submitted and the ecological risk assessment for Cry1F was updated most recently in 2010 (USEPA 2010b).

MON 87411 expresses Cry3Bb1 in addition to the DvSnf7 dsRNA. Cry3Bb1 is a delta-endotoxin from *Bt* subsp. *kumamotoensis* that has specific activity against insects within the Order Coleoptera. EPA determined that the Cry3Bb1 insecticidal protein expressed by MON 87411 is biochemically and functionally equivalent to the Cry3Bb1 expressed in MON 88017 corn (USEPA 2015a). The Cry3Bb1 protein expressed in MON 88017 was also determined to be biochemically and functionally equivalent to Cry3Bb1 expressed in MON 863 maize, and both were determined to have no unreasonable adverse effects on nontarget organisms (USEPA 2010a). The ecological risk assessment for the seed increase registration of MON 87411 most recently discussed the ecological risks of MON 87411 and determined that the data developed for Cry3Bb1 in MON 88017 and MON 863 supported the risk assessment of this protein as expressed in MON 87411 (USEPA 2015b).

DAS-59122-7 produces the *Bt* strain PS149B1 Cry34Ab1 and Cry35Ab1 proteins to protect against coleopteran pests such as corn rootworm. The most recent risk assessment and complete description of data supporting its registration is available in USEPA (2010d).

B. Ecological Risk Assessment for MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn

To bridge the ecological effects and environmental fate data of the individual proteins associated with each parental event to a combined trait PIP product, the combined trait PIP must be demonstrated as biochemically and functionally equivalent to their respective parental PIP events. Biochemical equivalence is typically described in product analysis data submitted to support the product registration. The functional equivalence is established by demonstrating that the effects of the pesticidal mixture of the combined PIP product on a susceptible pest species are comparable to the effects of each PIP pesticidal substance tested individually. Additionally, data must be submitted to show that exposure is not increased due to greater expression of any PIP pesticidal substance in the combined trait hybrid compared to the single-event parental lines. These data were reviewed for MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn in (USEPA 2015a, USEPA 2016) and found to support bridging these data.

Interactions between the PIP pesticidal substances in the combined trait hybrid also must be assessed to support bridging to data developed on the individual PIPs. This can be done by comparing the larval mortality observed for the mixture with the predicted responses based on the bioassay of each PIP pesticidal substance individually via sensitive insect bioassays. If there is no greater mortality than expected over the range of concentrations in a sensitive pest species, it is likely that there will be no synergism of the mixture against non-target organisms, and the effect of a mixture on non-target organisms can be predicted from the effects of the individual proteins alone. Therefore, data developed on the individual PIPs can be bridged to support the

risk assessment on the combination. Data to evaluate the potential for synergism between the Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, and Cry34/35Ab1 proteins and the DvSnf7 dsRNA expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn is reviewed herein, and risk assessment conclusions discussed below are based on these data, protein expression data, and the data bridged from the other products.

1. Synergism Data

Several previously submitted studies have assessed the potential for interaction between a number of the combinations of the Cry proteins produced by MON 89034 x TC1507 x MON 87411 x DAS-59122-7. For lepidopteran active proteins, combined activity of the Cry1A.105 and Cry2Ab2 proteins produced by MON 89034 was shown to be additive in corn ear worm (*Helicoverpa zea*, CEW) and ECB bioassays (USEPA 2010c). Additionally, the combined activity of Cry1A.105 and Cry2Ab2 expressed by MON 89034 and Cry1F produced by TC1507 was also determined to be additive in a bioassay with ECB (USEPA 2009).

Testing with coleopteran active Cry proteins has also been performed. A corn rootworm bioassay showed that the combined activity of the Cry3Bb1 and Cry34/Cry35Ab1 proteins is additive. The combination of the coleopteran active Cry3Bb1 and Cry34/35Ab1 produced by MON 88017 x DAS-59122-7 also did not alter the combined activity of the Cry1A.105, Cry2Ab2 and Cry1F in a bioassay with ECB (USEPA 2009).

Data from three studies are presented below that examined the potential for synergism between the Cry3Bb1 and DvSnf7 expressed in MON 87411 using assays with SCR and CPB. Another study was submitted to confirm that the combined activity of the lepidopteran active Cry1A.105/Cry2Ab2 and Cry1F proteins was additive within the mixture expressed by MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn using ECB. A fifth study is also available to evaluate the potential for a synergistic interaction between DvSnf7 and the combination of Cry34/35Ab1 and Cry3Bb1, and also evaluate whether a mixture of Cry1A.105, Cry2Ab2, and Cry1F, at a level that approximates expression in early season root tissue, alters the combined activity of DvSnf7, Cry34/35Ab1 and Cry3Bb1. These new studies are summarized in Table 4, and in individual descriptions of the studies that follow.

Table 4. Summary of synergism data submitted for MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn to support bridging data to individual PIP events.

Data Requirement	OCSPP Guideline	Test Substance	Results Summary and Classification	MRID No.
Interaction of Cry3Bb1 and DvSnf7, Southern corn rootworm, *Diabrotica undecimpunctata howardi*	N/A	*In vitro* transcribed DvSnf7 and microbially produced Cry3Bb1	No difference in toxicity (LC_{50}) was observed in SCR exposed to a lethal concentration of Cry3Bb1 combined with a sublethal concentration (EC_{10} for growth inhibition) of DvSnf7 DsRNA or vice versa. Testing with additional concentrations is necessary to confirm the potential for synergism. **Classification: Supplemental**	49505803
Interaction of Cry3Bb1 and	N/A	*In vitro* transcribed DvSnf7 and	Cited literature. No difference in growth inhibition (GI) observed in SCR exposed to combined concentrations testing equipotent	Levine et al. 2015

Data Requirement	OCSPP Guideline	Test Substance	Results Summary and Classification	MRID No.
DvSnf7, Southern corn rootworm, *Diabrotica undecimpunctata howardi*		microbially produced Cry3Bb1	concentrations of DvSnf7 and Cry3Bb1 at GI_{35}, GI_{50}, and GI_{65} levels for the individual proteins. **Classification: Acceptable**	
Interaction of Cry3Bb1 and DvSnf7, Colorado potato beetle, *Leptinotarsa decemlineata*	N/A	*In vitro* transcribed DvSnf7 and microbially produced Cry3Bb1	The toxicity of Cry3Bb1 protein to *L. decemlineata* does not change when exposed either to a lethal concentration of Cry3Bb1 alone or simultaneously exposed to Cry3Bb1 and 1000 ng DvSnf7 dsRNA/g diet. **Classification: Acceptable**	49315121
Interaction of Cry1A.105, Cry2Ab2, Cry1F with DvSnf7, Cry3Bb1 and Cry34/35Ab1, European corn borer *Ostrinia nubialis*	N/A	Lyophilized leaf material from MON 89034, TC1507, and MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn	Growth inhibition (GI_{50}) of ECB exposed to diet containing a range of concentrations of MON 89034 x TC1507 x MON 87411 x DAS-59122-7 combined trait corn leaf material did not differ from the value predicted from MON 89034 and TC1507 single trait corn. This study confirms that activity of the proteins expressed by these events are additive, and are not impacted by the presence of the coleopteran active PIPs. **Classification: Acceptable**	49781806
Interaction of DvSnf7, Cry3Bb1 and Cry34/35Ab1 and interaction of these three PIPs with a combination of Cry1A.105, Cry2Ab2, and Cry1F, Southern corn rootworm, *Diabrotica undecimpunctata howardi*	N/A	*In vitro* transcribed DvSnf7 and microbially produced Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, and Cry34/35Ab1	Growth inhibition (GI_{50}) of SCR exposed to diet containing a range of concentrations of DvSnf7, Cry3Bb1, and Cry34/35Ab1 did not differ from the value predicted from DvSnf7 alone and a mixture of Cry3Bb1 and Cry34/35Ab1. There was also no indication of synergism when SCR were tested with a combination of DvSnf7, Cry3Bb1, and Cry34/35Ab1 against a combination of Cry1A.105, Cry2Ab2, and Cry1F at a fixed concentration approximating early season root expression levels. **Classification: Acceptable**	49781805

Synergism – Cry3Bb1 and DvSnf7 in MON 87411

Synergism in SCR

This study tested the potential for synergism in the sensitive insect species, southern corn rootworm (MRID 49505803). The study was conducted as a 12-day bioassay. The potential for interaction between DvSnf7 and Cry3Bb1 was evaluated by comparing the LC_{50} values of a lethal level of Cry3Bb1 in the presence of DvSnf7 at a fixed sub-lethal concentration and a lethal level of DvSnf7 in the presence of Cry3Bb1 at a fixed sub-lethal concentration. The fixed sub-lethal concentration for DvSnf7 and the Cry3Bb1 protein used in the two binary mixtures was equivalent to an EC_{10} value for growth inhibition determined in method development bioassays. Cry3Bb1 and DvSnf7 were also tested at lethal levels alone (not as part of a binary mixture). Assay and buffer controls were included, and mortality was low (7% - 11%). The SCR concentration responses for the Cry3Bb1 protein alone and the Cry3Bb1 protein in the presence of a fixed sub-lethal concentration of DvSnf7 were nearly identical and this is reflected by

comparable LC_{50} values for the Cry3Bb1 protein alone and the binary mixture (LC_{50}= 46.28 µg/ml diet for Cry3Bb1 alone and 40.65 µg/ml for Cry3Bb1 combined with DvSnf7 at a fixed sub-lethal concentration). The SCR concentration responses for the DvSnf7 alone and DvSnf7 combined with Cry3Bb1 at a fixed sublethal concentration were also very comparable (LC_{50}= 6.35 ng/ml diet for DvSnf7 alone and 8.16 ng/ml diet for DvSnf7 combined with the sublethal concentration of Cry3Bb1). The study authors concluded that these data show a lack of synergism between DvSnf7 and Cry3Bb1.

EPA had previously raised concerns about the use of the fixed sublethal concentration that was stated to be the limit at which overt toxic signs would be observed. Monsanto Company justified this approach by citing to studies in which fixed sublethal concentrations have been used successfully to assess for and characterize synergistic interaction, including Tabashnik (1992) and citations within, as well as Belden and Lydy (2006). These papers do describe experiments in which fixed sublethal concentrations have been used, even in very small amounts (e.g., MacIntosh et al. [1990], cited in Tabashnik [1992]). However, these papers also describe additional testing at multiple concentrations, determination of synergism based on observations of sublethal effects, and/or adjustments in the target mortality in the component present in higher concentration (e.g., reduced to 10-20% in MacIntosh et al. [1990]). These approaches better characterize the nature of the potential interaction, and better ensure that an effect would be observed if it were to occur. While the approach used in this study is not unlike some of these other studies, and may be valid, there is still uncertainty as to whether a synergistic effect on mortality could be observed at the sublethal levels of DvSnf7 or Cry3Bb1 tested. Additional testing using sublethal endpoints (e.g., growth inhibition) or testing both Cry3Bb1 and DvSnf7 at multiple concentrations or median lethal levels would reduce this uncertainty. This study was originally submitted to support the seed increase registration of MON 87411, and was classified as supplemental. EPA required an additional study that was better designed to detect synergism between Cry3Bb1 and DvSnf7 for a full commercial registration involving MON 87411.

The additional data required are available in Levine et al. (2015), which was cited in support of this registration. This paper includes the work described in MRIDs 49505803 and 49315121, but also describes a study to determine the effect of the combination of DvSnf7 and Cry3Bb1 on growth inhibition in SCR. Bioassays were conducted to test three equipotent combinations of DvSnf7 and Cry3Bb1, which were selected from concentration response profiles on this endpoint that were conducted previously, in addition to separately testing the individual treatments at these levels. The three combinations were included to test effects at levels that resulted in 35%, 50%, and 65% growth inhibition for the individual treatments. A response addition model was used to predict the response to the mixture based on the response to the individual treatments, and observed values were compared to predicted values. The data presented in the paper showed somewhat higher levels of growth inhibition in the combined treatments of DvSnf7 and Cry3Bb1 (whether these were significantly higher was not explained), but there was good agreement (the paper states $p > 0.050$) between the observed and predicted effects, indicating that the effects of the combination were additive.

Synergism in CPB

An additional confirmatory study was submitted to evaluate the potential for interaction between the DvSnf7 and Cry3Bb1 protein using CPB in a 12-day continuous feeding bioassay (MRID 49315121). The Colorado potato beetle is not susceptible to DvSnf7, according to Bachman, et al. (2013). The potential for interaction was evaluated by comparing the LC_{50} values and the 95% confidence intervals of the Cry3Bb1 protein in the presence and absence of DvSnf7 at a fixed concentration of 1000 ng/mL diet. Nominal test concentrations for the Cry3Bb1 protein were based on the results of previous bioassays performed at the testing facility. Mean mortality in all assay and buffer controls was 10.42% at assay termination. Colorado potato beetle demonstrated nearly identical concentration-effect relationships to the Cry3Bb1 protein alone and in the presence of fixed concentration of DvSnf7. The study is acceptable as a confirmatory study of no interaction in a nontarget species, but does not definitively test interaction because it is unclear whether CPB has any sensitivity to DvSnf7.

Synergism –DvSnf7 with Cry Proteins in MON 89034 x TC1507 x MON 87411 x DAS-59122-7

Synergism in ECB

Seven-day ECB bioassays were performed with lyophilized maize leaf tissue from either single trait MON 89034 or TC1507 corn or combined trait MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn to test whether the interaction of proteins expressed in MON 89034 and TC1507 are additive when expressed in the combined trait corn (MRID 49781806). The lyophilized leaf tissue from each line of corn was ground into a fine powder and incorporated into diet over a concentration range. Each test and control substance was assayed concurrently in three replicate bioassays. Growth inhibition (GI) was the response variable that was used for concentration-response modeling, and the GI_{50} value was selected as the endpoint for comparison using a concentration addition model. In each bioassay, ECB demonstrated concentration-dependent responses for growth inhibition. GI_{50} values for MON 89034 and TC1507 were 0.022 mg tissue/ml diet and 0.64 mg tissue/ml diet, respectively, which were used in the model to determine a predicted GI_{50} value for the bioassay with the combined trait corn. The observed GI_{50} value for MON 89034 x TC1507 x MON 87411 x DAS 59122-7 from diet bioassays was 0.022 mg tissue/ml diet with a 95% confidence interval (CI) 0.017 to 0.026 mg tissue/ml diet. The predicted GI_{50} value was 0.021 mg tissue/ml diet, which was comparable to the observed value and was captured within the 95% CI (0.017 to 0.026 mg tissue/ml diet) for the estimated GI_{50} value. These results confirm that there is no interaction between the Cry proteins expressed in MON 89034 and TC1507 corn when expressed in the combined trait corn, and that this result is not affected by the presence of the coleopteran active PIPs, including DvSnf7. EPA determined that this study was acceptable; however, noted that mortality was only reported for the controls (ranging from 0-3%) and not the other treatments. While there is no reason to expect mortality in the other treatments, data to show whether mortality was equivalent for the other treatments should be provided.

Synergism in SCR

An additional study (MRID 49781805) was submitted to test the potential for synergistic interaction between DvSnf7 and the combination of Cry34/35Ab1 and Cry3Bb1. The study also tested whether a mixture of Cry1A.105, Cry2Ab2, and Cry1F at a concentration that approximated expression in early season root tissue, alters the combined activity of DvSnf7, Cry34/35Ab1 and Cry3Bb1. Twelve-day SCR bioassays were performed with *in vitro* transcribed DvSnf7 and bacterially expressed Cry3Bb1, Cry34/35Ab1, Cry1A.105, Cry2Ab2, and Cry1F incorporated into diet over a concentration range. Bioassays tested ranges of concentrations involving:

1) DvSnf7 alone
2) A mixture of Cry3Bb1 and Cry34/35Ab1
3) A mixture of DvSnf7, Cry3Bb1 and Cry34/35Ab1
4) A mixture of Cry1A.105, Cry2Ab2 and Cry1F at a concentration approximating expression of these three proteins in early season root tissue
5) A mixture of DvSnf7, Cry3Bb1 and Cry34/35Ab1 at multiple concentrations in the presence of a mixture of Cry1A.105, Cry2Ab2 and Cry1F at a fixed concentration.

Growth inhibition (GI) was the response variable used for concentration-response modeling, and comparisons of predicted (determined using the concentration addition model [Finney 1971, Tabashnik 1992]), and observed GI_{50} values were used to test the hypothesis for comparisons #3 and #5 (#1 and #2 were used in the model to determine predicted values; #4 was a control treatment).

The control treatment had no significant effect on SCR growth in the 12-day assays. Additivity for the combination of DvSnf7, Cry3Bb1 and Cry34/35Ab1 (#3 above) was concluded based on the overlap of 95% confidence intervals (CIs) for the predicted and estimated GI_{50} values (CI for predicted = 4.7 to 7.0 µg/ml diet; CI for estimated = 5.9 to 8.9 µg/ml diet). The predicted GI_{50} value (5.9 µg/ml diet) is contained just within the lower end of the 95% CI for the estimated GI_{50} value for the mixture of DvSnf7, Cry3Bb1 and Cry34/35Ab1 (5.9 to 8.9 µg/ml diet), which is indicative of additivity (Tabashnik 1992, Jonker et al. 2012).

For comparison #5, GI_{50} values were comparable for the mixture of DvSnf7, Cry3Bb1 and Cry34/35Ab1 with and without the addition of a fixed concentration of the Cry1A.105, Cry2Ab2 and Cry1F proteins (7.1 versus 7.4 µg/ml diet). The 95% CIs for the mixture of DvSnf7, Cry3Bb1 and Cry34/35Ab1 with and without the addition of the three lepidopteran active proteins greatly overlap (5.4 to 8.7 µg/ml versus 5.9 to 8.9 µg/ml). Furthermore, the predicted GI_{50} value of 5.9 µg/ml diet for the mixture of DvSnf7, Cry3Bb1 and Cry34/35Ab1 is contained within the 95% CI (5.4 to 8.7 µg/ml diet) of the estimated GI_{50} value. These results show that the addition of the Cry1A.105, Cry2Ab2 and Cry1F proteins at a fixed concentration does not alter additivity between DvSnf7 and the combination of Cry3Bb1 and Cry34/35Ab1.

Based on the results, effects of DvSnf7 and the combination of Cry3Bb1 and Cry34/35Ab1 against a target pest species are additive, and the presence of Cry1A.105, Cry2Ab2 and Cry1F at

representative expression levels *in planta* does not alter the combined activity of DvSnf7, Cry3Bb1 and Cry34/35Ab1. EPA determined that this study is acceptable.

2. Ecological Risk Conclusions for MON 89034 x TC1507 x MON 87411 x DAS-59122-7

Nontarget Organism Risks

Since expression levels of Cry1A.105, Cry2Ab2, Cry1F, DvSnf7, Cry3Bb1 and Cry34/35Ab1 do not exceed those previously considered in ecological risk assessments for each of these individual PIPs, and because data indicate no potential synergism between them, the nontarget risk conclusions drawn for each of these PIPs individually also apply to their combination in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn.

Acceptable data has shown that adverse effects are not expected for each of these PIPs, based on the Biopesticide Registration Action Documents cited above (USEPA 2010a-d) and also based on the data and other information for DvSnf7 discussed in Section II above. To date, no concerns for adverse effects have been identified for nontarget organisms potentially exposed to any of the individual PIPs in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn. Therefore, EPA concludes that the combination of PIPs as expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn are not expected to cause adverse effects to nontarget organisms.

Outcrossing and Development of Invasiveness

EPA has previously determined that there is no significant risk of gene flow and introgression of any *Bt* endotoxin trait by wild or weedy relatives of corn in the U.S., its possessions or territories (see extensive discussion in U.S. EPA 2010a-b). Since these conclusions are based on the nature of pollination, survival of hybrid offspring, and development of invasiveness in corn and its relatives, these conclusions would apply to all of the Cry proteins and also DvSnf7 expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn.

Endangered Species Conclusions

EPA has determined that DvSnf7 as expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn is unlikely to have adverse effects on nontarget organisms. Therefore, a "No Effect" determination is made for direct and indirect effects to all federally listed threatened and endangered ("listed") species and their designated critical habitats.

Because the Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, and Cry34/35Ab1 proteins expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn are selective for either coleopteran or lepidopteran species, any adverse effects to listed species other than insects within those taxonomic orders are unlikely. Therefore, a "No Effect" determination is made for direct and indirect effects to all other listed species and their designated habitats, and the endangered species assessment for MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn is thus focused on potential direct effects to listed coleopteran and lepidopteran species.

Exposure to coleopteran and lepidopteran species is expected to be limited to direct consumption of MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn, including incidental exposure to pollen that may be deposited within the margins around corn fields planted with MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn. Therefore, since most listed coleopteran and lepidopteran species are habitat specialists and do not utilize corn or corn fields as habitat, many can be eliminated from consideration because exposure is not anticipated to occur.

EPA's most recent assessment of the potential risks to listed threatened or endangered coleopteran and lepidopteran species was addressed in relation to the most recent new PIP (other than DvSnf7) registered in corn (USEPA 2012). Updates have been completed for registrations of new PIP combinations in corn, but have largely remained unchanged. EPA most recently updated the assessment for coleopteran species for the MON 87411 seed increase registration (USEPA 2015b). Thus far, EPA has made "No Effect" determinations for direct and indirect effects to listed coleopterans and lepidopterans and their designated critical habitats for Cry proteins expressed in corn. Conclusions drawn in those assessments would also apply to the assessment for MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn. Since these assessments, additional lepidopteran and coleopteran species have been added for consideration, and further analysis is required for this proposed registration of MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn. This analysis is currently underway, and EPA will update this endangered species assessment prior to making a registration decision.

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